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(54) Title: COUP-TFI DIRECTED THERAPIES IN THE TREATMENT OF DISEASE

(57) **Abstract**

Methods for the treatment of neurological diseases, bone disease, and hearing and balance diseases are provided. These comprise the step of administering an antagonist to COUP-TFI in a therapeutic effective amount. The antagonist to COUP-TFI can be used to also prevent degeneration of bone, and nerves and neurons. Additionally, a method includes the introduction of the COUP-TFI gene into the mammal wherein said introduced gene can be induced by an agonistic to COUP-TFI to enhance the growth of nerve tissue, bone tissue and inner ear tissue.

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COUP-TFI DIRECTED THERAPIES IN THE TREATMENT OF DISEASE

Background of the Invention

1. Field of the Invention

The present invention relates generally to the field of molecular endocrinology and receptor pharmacology. More specifically, it relates to the use of agonists and antagonists of the COUP-TFI receptor for nerve regeneration, prevention of nerve degeneration, treatment of neurodegenerative, learning and memory disorders, bone-related disorders, and hearing and balance related disorders.

2.Description of the Related Art

The steroid/thyroid hormone receptor superfamily of proteins consists of many ligand-activated transcriptional regulators and numerous orphan receptors. Among the orphan receptors, COUP-TF (Chicken Ovalbumin Upstream Promoter-Transcription Factor) is one of the most characterized orphan receptors of the steroid/thyroid hormone receptor superfamily.

COUP-TF was first identified as a homodimer that binds to a direct repeat regulatory element in the chicken ovalbumin promoter. This element contains an imperfect direct repeat of AGGTCA sequence, which has been shown to be essential for efficient *in vitro* transcription. Several years ago, the inventors of the present Invention successfully purified COUP-TF from HeLa cell nuclear extracts using a combination of

cell cDNA library. A clone that cross-reacted with both the antibody and the COUP-TF binding site was obtained and designated as human COUP-TFI (hCOUP-TFI). Sequence analysis revealed that COUP-TFI is a member of the steroid/thyroid superfamily.

Two COUP-TF genes were initially cloned from human cells, named hCOUP-TFI and hCOUP-TFII. COUP-TFII is the subject of a co-pending U.S. Application Serial No. 09/097,725 filed June 16, 1998, entitled "COUP-TFII: A Nuclear Receptor Required for Angiogenesis." This was originally filed as a Provisional Application on June 18, 1997, and both are hereby incorporated by reference into the present Application. In addition, a thorough review of COUP-TF is provided in Tsai, S. and Tsai, M.-J., *Chick Ovalbumin Upstream Promoter-Transcription Factor (COUP-TFs): Coming of Age*, 18 Endocrinology Rev. 229 (1997). Prior work by the inventors of the present invention is also seen in Wang, L.-H. *et al.*, *The COUP-TFs compose a family of functionally related transcription factors*, 1 Gene Expression 207 (1991); Ritchie, H. H. *et al.*, *COUP-TF gene: a structure unique for the steroid/thyroid receptor superfamily*, 18 Nucleic Acid Res. 6857 (1990); and Wang, L.-H., *COUP transcription factor is a member of the steroid receptor superfamily*, 340 Nature 163 (1989).

Since the initial cloning, the COUP-TF subfamily has expanded rapidly. The amino-acid sequence deduced from the hCOUP-TF cDNAs reveal significant similarities to members of the steroid/thyroid superfamily of genes.

Homologues have been cloned from many species, for example from *Drosophila* to mouse. Most of the species examined have more than one COUP-TF homologue. In addition, COUP-TF subfamily members share a high degree of homology within and between species, implying that

signify the presence of the DNA-binding domain (DBD) of a nuclear receptor. In COUP-TFs, all 20 invariant amino acids were conserved, and 11 of 12 conserved residues were identical except that a conserved lysine in the second finger is replaced by a glutamine. Based on the P-box sequence, COUP-TFs can be classified as members of the estrogen receptor subfamily, which bind to a Pu-GGTCA repeat. There is only one amino acid difference within the DBD of COUP-TFI and II, which is a conservative change from Ser to Thr.

It has been demonstrated that COUP-TFs bind to AGGTCA direct repeats with various spacings, which include the response elements for the retinoic acid receptors (RAR), retinoid X receptors (RXR), vitamin D_3 receptor (VDR), and thyroid hormone receptors (TR). When cotransfected, COUP-TFs can inhibit the activation function of the above mentioned receptors in the presence of their cognate ligands. COUP-TFs exert their inhibitory activity mainly by competitive DNA binding of the common response elements and by heterodimerizing with the common partner RXR. Therefore, COUP-TFs are proposed to modulate vitamin D_3, the thyroid hormone, and the RA signaling pathways. In addition, COUP-TFs have been shown to negatively regulate the expression of many genes by competing for the same or overlapping response elements with other positive regulators. For instance, COUP-TFs could negatively regulate the expression of several apolipoproteins by antagonizing the positive regulator HNF-4. Thus, COUP-TFs may also function independently of the above mentioned signaling pathways.

In *Drosophila*, mutation of the COUP-TF homologue, the seven-up gene (svp), is lethal. Mosaic analysis demonstrated that svp activity is required for

the R7 cell. In zebrafish, Xenopus, chick and mouse, COUP-TFs are expressed at high levels in the developing central nervous system, suggesting that they are involved in neurogenesis. In mouse, COUP-TFs are also highly expressed in many developing organs, and the expression level decreases upon completion of differentiation, suggesting that COUP-TFs may also be involved in organogenesis. Collectively, this evidence suggests that COUP-TFs play conserved and vital roles during embryonic development.

mCOUP-TFI and mCOUP-TFII share an exceptional degree of homology at the amino acid level (99% identity in the DNA-binding domain and 96% identity in the putative ligand-binding domain).

Relatively non-selective DNA binding of COUP-TFs are expected to down-regulate the hormonal induction of target genes by VDR, TR, and RAR. Indeed Cotransfection of COUP-TFI expression vectors inhibited the hormonal induction of VDR-, TR-, and RAR-dependent activation of reporter gene activity.

VDR, TR, and RAR have been demonstrated to activate target genes containing DR3, DR4 and DR5 response elements, respectively. By virtue of their promiscuous DNA binding, COUP-TFs are expected to downregulate the hormonal induction of target genes by VDR, TR, and RAR. Cotransfection of COUP-TFI or II expression vectors inhibited the hormonal induction of VDR-, TR-, and RAR-dependent activation of reporter activity. The inhibition of transcriptional activity by COUP-TFs is dose-dependent, as the reporter activity is progressively inhibited by increasing concentrations of the transfected COUP-TF expression vector. COUP-TFs not only inhibit the hormone response of reporters

thyroid response element (TRE), and retinoic acid response element (RARE) sequences as in the respective cases of the osteocalcin, myosin heavy chain, and BRAR promoters. In addition, COUP-TFs have been shown to antagonize the HNF4-dependent transcriptional activation of many liver-specific genes and to suppress OCT3/4 expression during retinoid-induced differentiation of P19 embryonic carcinoma cells. The mechanisms by which COUP-TFs inhibit the transactivation of other members of the steroid receptor superfamily are described in Tsai, S. and Tsai, M.-J., *Chick Ovalbumin Upstream Promoter-Transcription Factor (COUP-TFs): Coming of Age*, 18 Endocrinology Rev. 229 (1997).

Yet despite this detailed knowledge regarding the molecular mechanism of COUP-TFI, the precise physiological role of COUP-TFs as activators has been, until now, unclear. Numerous factors frustrate this effort; for instance, some of the activity has been observed only when a response element was analyzed out of the context of its promoter. In addition, inducibility is low in general, and it is only observed in a few particular cell types.

Thus, the present Invention is based on fundamental new research which uncovers many aspects of COUP-TFI physiology. This knowledge in turn has led to the creation of novel therapies.

Summary of the Invention

An object of the present invention is a method for enhancing of nerve regeneration in a mammal.

An additional object of the present invention is a method for the prevention of nerve or neuron degeneration in a mammal.

Another object of the present invention is a method for enhancement of learning and memory.

An object of the present invention is a method for treatment of learning and memory diseases.

An additional object of the present invention is a method for prevention of loss of hearing.

An additional object of the present invention is a method for the control of motion sickness.

A further object of the present invention is a method for the prevention of bone loss in mammals.

An additional object of the present invention is a method for the regeneration of bone formation in mammals.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention a method for treating neurodegenerative diseases in a mammal, comprising administering to a mammal affected with said neurodegenerative disease a therapeutic effective amount of an agonist of COUP-TFI, wherein said agonist induces growth of neurological tissue.

In specific embodiments of the present invention the neurodegenerative disorder is selected from the group consisting of Alzheimer's Disease, Huntington's Disease, seizure, Parkinson Disease, stroke, multiple sclerosis, and learning and memory defects.

Another embodiment of the present invention includes a method of enhancing nerve regeneration in a mammal comprising the step of administering to a mammal an effective amount of an agonist of COUP-TFI, wherein said agonist induces the growth of nerves or neurons.

In specific embodiments of the present invention, said agonist induces or stimulates the growth of the axonal projection.

In specific embodiments of the present invention, the agonist stimulates the growth of the axonal projection between the glossopharyngeal ganglion in the hindbrain.

In specific embodiments of the present invention, the agonist stimulates growth of the IX ganglion.

A further embodiment of the present invention includes a method for the prevention of nerve and neuron degeneration in mammals comprising the step of administering to a mammal a therapeutically effective amount of an agonist of COUP-TFI, wherein said agonist enhances nerve or nerve growth.

An additional method of the present invention is a method of treating hearing defects comprising the step of administering to a mammal affected with said hearing defect a therapeutic effect amount of an agonist to COUP-TFI, wherein said agonist induces growth of the inner ear.

An additional embodiment of the present invention includes a method of treating balance defects in a mammal comprising the step of administering to a mammal affected with said balance defects therapeutic effective amount of an agonist to COUP-TFI, wherein said agonist induces growth in the inner ear.

Another embodiment of the present invention includes a method of inducing regeneration of bone formation in a mammal comprising the step of administering to said mammal a therapeutic effective amount of agonist of COUP-TFI, wherein said agonist induces bone formation.

Another embodiment of the present invention includes a method for prevention of bone loss in a mammal an effective amount of an agonist of COUP-TFI, wherein said agonist inhibits the loss of bone.

with an injured inner ear a therapeutic effective amount of an agonist to COUP-TFI, wherein said agonist induces growth or inhibits degeneration of the inner ear.

An additional embodiment of the present invention includes a method for enhancing the growth of the cochlear duct, scala tympani or sacculus in a mammal comprising the step of administering a therapeutic effective amount of an agonist to COUP-TFI, wherein said agonist induces growth.

Another embodiment of the present invention includes a method for treating neurodegenerative diseases in a mammal comprising the step of introducing by genetic therapy a COUP-TFI gene operatively linked to a promoter, wherein said COUP-TFI gene is inducible by the addition of an agonist and said expression of COUP-TFI enhances expression of COUP-TFI increasing the growth of neural and neuronal tissue.

Another embodiment of the present invention includes a method for treating bone diseases in a mammal comprising the step of introducing by genetic therapy a COUP-TFI gene operatively linked to a promoter, wherein said COUP-TFI gene is inducible by the addition of an agonist and said expression of COUP-TFI enhances expression of COUP-TFI increasing the growth of bone tissue.

An additional embodiment of the present invention includes a method for treating hearing and balance diseases in a mammal comprising the step of introducing by genetic therapy a COUP-TFI gene operatively linked to a promoter, wherein said COUP-TFI gene is inducible by the addition of an agonist and said expression of COUP-TFI enhances expression of COUP-TFI increasing the growth of inner ear tissue.

are given for the purpose of disclosure, when taken in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 depicts targeted disruption of the mCOUP-TFI locus. The mCOUP-TFI locus is shown on top with exons I-III in boxes. The targeting vector and targeting locus are shown below.

Figures 2A and 2B show a representative litter from an mCOUP-TFI heterozygote intercross as seen on Southern blot (Fig. 2A) and Northern blot (Fig. 2B) analysis.

Figures 3A through 3D depict whole-mount immunohistochemistry on wildtype and homozygote embryos using 2H3 anti-neurofilament antibody showing the progression of cranial ganglion formation (dorsal is to the left, ventral is to the right, scale bar, 100 μm). Figures 3A and 3C depict the wildtype; Figure 3B and 3D depict mutant embryos at E9.5 and E10.5, respectively. Figure 3 also shows the ganglion IX in two different stages of development.

Figures 4A through 4F depict the expression of mCOUP-TFI. Figures 4A through 4C show whole mount *in situ* hybridization of mCOUP-TFI in E8.0 (Figure 4A), 8.5 (Figure 4B), and 9.0 (Figure 4C) embryos, respectively. Figures 4D through 4F show sections of a whole-mount stained E8.5 embryo.

Figures 5A through 5F show the expression of rhombomere specific genes in mCOUP-TFI mutants. Whole-mount *in situ* hybridization was performed on wildtype

Figures 6A through 6F depict an analysis of cranial NCC migration in mCOUP-TFI mutants. Wildtype (Figs. 6A, 6C and 6E) or mutant embryos (Figs. 6B, 6D and 6F) were hybridized with CRABP I antisense probe to examine the migration of the neural crest cells. Whole-mount *in situ* hybridization on E9.0 (Figs. 6A and 6B) and E9.5 (Figs. 6C through 6F) embryos. Figures 6E and 6F: *in situ* hybridization with CRABP I antisense probe on sections of late E9.5 embryos.

Figures 7A through 7D show the developmental progression of the formation of the IXth ganglion as visualized by X-gal staining.

Figures 8A through 8H depict excessive cell death in mCOUP-TFI mutant embryos. mCOUP-TFI wildtype (Figs. 8A through 8D) and mutant (Figs. 8E through 8H).

Figures 9A through 9I show whole-mount analysis of axonal projections (scale bars in Figures 9A through 9C, 100 μ; E-H, 50 μm; Figure 9D, 20 μm). Multiple defects in cranial nerve projections are detected in severely affected embryos. Figure 9A shows wildtype E10.5 embryo. The right (Fig. 9B) and the left (Fig. 9C) side of an E10.5 mutant embryo. Figure 9D through 9H show enlargement of cranial nerve IX-XII region from Figures 9B and 9C. Figure 9I shows an enlargement of wildtype oculomotor nerve.

Figures 10A through 10D show whole-mount immunohistochemistry of wildtype (Figs. 10A and 10C) and mutant E11.5 fetuses (Figs. 10B and 10D) using 2H3 anti-neurofilament antibody. Figure 10A and Figure 10B show sagittal views at the level of posterior hindbrain

Figures 11A through 11F show whole-mount views of alizarin red (mineralized bone) and alcian blue (cartilage) stained skulls of newborns (Figs. 11A through 11D) and 17 day fetuses (Figs. 11E and 11F) of wildtype (Fig. 11A), heterozygotes (Figs. 11C and 11E) and COUP-TFI mutants (Figs. 11B, 11D, and 11F).

Figures 12A through 12D show whole-mount views of cervical vertebrae in wildtype (Figs. 12A and 12B) and COUP-TFI mutants (Figs. 12C and 12D).

Figures 13A through 13D show whole-mount views of wildtype (Fig. 13A, +/+) and COUP-TFI mutant (Fig. 13C, -/-) cochlea showing approximately 2.5 turns of cochlear duct (line is drawn through the middle of the cochlear duct) in wildtype, but less than 2 turns in mutant. Figures 13B and 13D show haematoxylin- and eosin-stained sagittal view through the mid-mediolar plane of wildtype (Fig. 13B) and mutant (Fig. 13D) cochlea. The following symbols are used: basal coil (BC), median coil (MC), and apical coil (AC) of the cochlear ducts.

Figures 14A through 14H show whole-mount and sectional views of alizarin red (mineralized bone) and alcian blue (cartilage) stained wildtype (Figs. 14A through 14D) and COUP-TFI mutant (Figs. 14E through 14H) inner ears showing defects in formation of the sacculus. The following symbols are used: U utriculus, S sacculus, SC semicircular canals, C cochlea, M malleus, I incus, ST stapes, T tympanic ring.

Figures 15A through 15D shows that the cortex of

mouse brains. Figures 15A and 15B show the cortex (cp) in the (-/-) is thinner than that in the (+/+), especially at the dorsal lateral regions. The cortical subplate (sp) is hardly visible in the (-/-). Figure 15C and 15D represents higher magnification of the cortical regions containing the subplate which is poorly formed in the (-/-) as compared to (+/+). cc, corpus callosum; cp, cortex; c-p, caudate-putamen; sn, septal nucleus; sp, subplate.

Figure 16A through 16D show that the cortical subplate is greatly reduced in the cortex of COUP-TFI null mice. Coronal sections of E17.5 mouse brains are immunohistochemically stained with an antibody against GAP43, a marker for the growth cone of axon. GAP43 expression is detected in the subplate and intermediate zone of the cortex of (Figs. 16A and 16C, +/+). However, in the cortex of (Figs. 16B and 16D, -/-), GAP43 only stains the intermediate zone. The subplate in (Figs. 16B and 16D, -/-) appears to be missing which is consistent with histological analysis. Figures 16C and 16D are the bright-field view of coronal sections counterstained with H&E.

Figures 17A through 17D show that the cerebral cortical layers of COUP-TFI null mice are poorly differentiated. These are coronal sections of 3-week old mouse brains. Figures 17C and 17D are the higher magnification of the upper panels. The (-/-) cerebral cortex is thinner and the cortical layers are poorly differentiated. The layer IV in (-/-) appears to be missing, while the white matter (WM) layer is thicker in (-/-) than in (+/+). I-VI, cortical layer I through VI.

sections of 3-week old mouse brains are stained with luxol fast blue and counterstained with nuclear fast red. Figures 18C and 18D are the higher magnification of the white matter of the Figures 18A and 18B. Figures 18E and 18F are the higher magnification of the caudate-putamen of the Figures 18A and 18B. The axon bundles in both white matter and caudate-putamen of (-/-) are less myelinated indicated by blue staining.

Figures 19A through 19F show that myelin basic protein expression is downregulated in the cortex of COUP-TFI null mice. Coronal sections of 3-week old brains are immunohistochemically stained with an antibody against myelin basic protein. Figures 18C and 18D are the higher magnification of the white matter area shown on the Figures 18A and 18B. Figures 18E and 18F are the higher magnification of the caudate-putamen area of the Figures 18A and 18B. Expression of myelin basic protein in both white matter and caudate-putamen of (-/-) are greatly reduced.

Figures 20A through 20D show that Tst-1/SCIP/Oct-6 gene expression is downregulated in the cortex of COUP-TFI null mice. Figures 20C and 20D show *in situ* hybridization of coronal sections of P1 (+/-) and (-/-) mouse brains with antisense probe for Tst-1/SCIP/Oct-6. Figures 20A and 20B upper panels are the bright-field view of coronal sections counterstained with H&E.

The drawings are not necessarily to scale. Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

Detailed Description of the Preferred Embodiments

It is readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

The present Invention is directed to novel therapies based on a particular protein, COUP-TFI, i.e., "COUP-TFI directed therapies." This Application describes the newly discovered physiological role of this unusual protein, COUP-TFI, whose function is generally related to regulating transcription (e.g., the biosynthesis of proteins).

Based primarily upon studies involving mice in which the gene coding for the COUP-TFI protein has been deleted, and knowledge of related receptors, the function of COUP-TFI in mammalian physiology is described herein. As evidenced by the data and explanation contained herein, the physiological role of COUP-TFI is directed to at least three major areas: neurological development, bone morphogenesis, and inner ear development. Having determined the physiology of COUP-TFI, Applicants present herein novel therapies which exploit the biochemistry/molecular biology and physiological role of COUP-TFI to treat neurological disorders, learning and memory, bone-related disorders, and hearing-related disorders--all disorders known to occur in the absence of COUP-TFI. Hence COUP-TFI and its agonists are promising therapeutic agents for the types of maladies known to result from the absence of COUP-TF, and are also promising therapeutic agents for related disorders, whether or not caused by COUP-TF deficiency. For example, one therapeutic technique disclosed and claimed in the present Invention involves administering to a subject, afflicted with one of the

events, culminating eventually in a transcriptional response, i.e., the formation of a protein.

Definitions

"Agonist" is a compound which interacts with the COUP-TFI receptor to induce a transcriptional response.

"Antagonist" is a compound which, interacts with or binds to the COUP-TFI receptor and thereby inhibits the activity of a receptor agonist.

"Disease" or "Disorder" as used herein is generally consonant with the ordinary meaning to the skilled artisan: i.e., any deviation from or interruption of the normal structure or function of any part or system of the body.

"Orphan receptors" is a designation given to a series of cloned receptors whose primary sequence is closely related to the steroid hormone receptors, but for which no ligand has been described.

"Steroid/thyroid hormone receptor superfamily" is a classification of a group of proteins, some of which are known steroid receptors whose primary sequence suggests that they are related to each other.

"Transfected/Transfection" is a term describing the process of directly introducing DNA into a mammalian cell.

Therapeutic Effective Amount

As used in the present invention, the compound will be considered a therapeutic effective amount if it decreases, delays or eliminates the onset of a neurodegenerative disease. In addition, a compound will be considered therapeutic effective if it enhances nerve growth, learning and memory, bone formation or inhibits or slows down or delays nerve or bone degeneration or breakdown. Similarly, therapeutic effective shall also mean [illegible]

these cases the compound may not provide a cure but may only provide partial benefit. A physiological change having some benefits is considered therapeutically beneficial. Thus, an amount of a compound which provides a physiological change is considered an "effective amount" or a "therapeutic effective amount".

A compound or composition is said to be "pharmaceutically acceptable" if its administration can be tolerated by a recipient mammal. Such an agent is said to be administered in a "therapeutic effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a change in the physiology of a recipient mammal.

Dosage and Formulation

The agonists, antagonists, proteins for inducing expression of COUP-TFI, the COUP-TFI gene, and the COUP-TFI protein itself (active ingredients) of this invention can be formulated and administered to inhibit or decrease the symptoms of a variety of disease states (neurodegenerative, inner ear and bone disease) or enhance or improve the success of treatments (nerve growth, learning and memory, bone growth or inner ear growth) by any means that produces contact of the active ingredient with the agent's site of action in the body of a mammal. These compounds can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic active ingredients or in a combination of therapeutic active ingredients. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will be a therapeutic

pharmacodynamic characteristics of the particular active ingredient and its mode and route of administration; age, sex, health and weight of the recipient; nature and extent of symptoms; kind of concurrent treatment, frequency of treatment, and the effect desired. These relationships are generally known in the art for compounds having similar effects and can be readily determined by the skilled artisan.

Dosage (composition) suitable for internal administration in the treatment of proliferative disease generally contain from about 1 to about 500 mg of active ingredient per unit. In these pharmaceutical compositions, the active ingredient will ordinarily be present in an amount of about 0.05 to 95% by weight based on the total weight of the composition.

The active ingredient can be administered orally in solid dosage forms such as capsules, tablets and powders, or in liquid dosage forms such as elixirs, syrups, emulsions and suspensions. The active ingredient can also be formulated for administration parenterally by injection, rapid infusion, nasopharyngeal absorption or dermoabsorption. The agent may be administered intramuscularly, intravenously, or as a suppository. They can be given in divided doses or in sustained released form. Additionally, gene therapy may be used to target the compound. The skilled artisan can readily recognize that the dosage for this method must be adjusted depending on the efficacy of delivery.

Gelatin capsules contain the active ingredient and powdered carriers such as lactose, sucrose, mannitol, starch, cellulose derivatives, magnesium stearate, stearic acid, and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release

can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric coated for selective disintegration in the gastrointestinal tract.

Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient's acceptance.

In general, water, a suitable oil, saline, aqueous dextrose (glucose), and related sugar solutions and glycols such as propylene glycol or polyethylene glycols are suitable carriers for parenteral solutions. Solutions for parenteral administration contain preferably a water soluble salt of the active ingredient, suitable stabilizing agents and, if necessary, buffer substances. Antioxidizing agents such as sodium bisulfate, sodium sulfite or ascorbic acid, either alone or combined, are suitable stabilizing agents. Also used are citric acid and its salts, and sodium EDTA. In addition, parenteral solutions can contain preservatives such as benzalkonium chloride, methyl- or propyl-paraben, and chlorobutanol. Suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field.

Additionally, standard pharmaceutical methods can be employed to control the duration of action. These are well known in the art and include control-release preparations, and can include appropriate macromolecules, for example: polymers, polyesters, polyaminoacids, polyvinyl, pyrolidone, ethylenevinylacetate, methyl cellulose, carboxymethyl cellulose or protamine sulfate. The concentration of macromolecules as well as the methods of incorporation can be adjusted in order to control release.

ethylenevinylacetate copolymers. In addition to being incorporated, these agents can also be used to trap the compound in microcapsules.

Useful pharmaceutical dosage forms for administration of the compounds of this invention can be illustrated as follows.

Capsules: Capsules are prepared by filling standard two-piece hard gelatin capsulates each with 100 milligram of powdered active ingredient, 175 milligrams of lactose, 24 milligrams of talc and 6 milligrams magnesium stearate.

Soft Gelatin Capsules: A mixture of active ingredient in soybean oil is prepared and injected by means of a positive displacement pump into gelatin to form soft gelatin capsules containing 100 milligrams of the active ingredient. The capsules are then washed and dried.

Tablets: Tablets are prepared by conventional procedures so that the dosage unit is 100 milligrams of active ingredient. 0.2 milligrams of colloidal silicon dioxide, 5 milligrams of magnesium stearate, 275 milligrams of microcrystalline cellulose, 11 milligrams of cornstarch and 98.8 milligrams of lactose. Appropriate coatings may be applied to increase palatability or to delay absorption.

Injectable: A parenteral composition suitable for administration by injection is prepared by stirring 1.5% by weight of active ingredients in 10% by volume propylene glycol and water. The solution is made isotonic with sodium chloride and sterilized.

Suspension: An aqueous suspension is prepared for oral administration so that each 5 millimeters contain 100 milligrams of finely divided active ingredient, 200 milligrams of sodium carboxymethyl cellulose, 5 milligrams of sodium benzoate

In gene therapy, it is known that a variety of methods can be used by those skilled in the art. In gene therapy, the compounds can also be targeted to specific locations depending on the method used. Any other procedures for use in targeting of compounds can be used. In addition, the compounds can be applied topically or directly to the location in which the intervention is needed.

In addition if it is desired to administer to the subject, the COUP-TF gene directly, a biolistic device is preferably used, such as those described in Morikawa, H., *et al.*, *Transient expression of foreign genes in plant cells and tissues obtained by a simple biolistic device (particle-gun)*, 31 Appl. Microbiol. Biotechnol. 320 (1989).

One embodiment of the present invention includes a method for treating neurodegenerative diseases in a mammal, comprising the step of administering to a mammal affected with said neurodegenerative disease a therapeutic effective amount of an agonist of COUP-TFI, wherein said agonist induces growth of neurological tissue.

In specific embodiments of the present invention the neurodegenerative disorder is selected from the group consisting of Alzheimer's Disease, Huntington's Disease, seizure, Parkinson Disease, stroke, multiple sclerosis, and learning and memory defects.

Another embodiment of the present invention includes a method of enhancing nerve regeneration in a mammal comprising the step of administering to a mammal an effective amount of an agonist of COUP-TFI, wherein said agonist induces the growth of nerves or neurons.

In specific embodiments of the present invention, said agonist induces or stimulates the growth of the axonal [illegible]

In specific embodiments of the present invention, the agonist enhances or stimulates axonal differentiation and myelination.

In specific embodiments of the present invention, the agonist stimulates the growth of the axonal projection between the glossopharyngeal ganglion in the hindbrain.

In specific embodiments of the present invention, the agonist stimulates growth of the IX ganglion.

A further embodiment of the present invention includes a method for the prevention of nerve and neuron degeneration in mammals comprising the step of administering to a mammal a therapeutically effective amount of an agonist of COUP-TFI, wherein said agonist enhances nerve or nerve growth.

An additional method of the present invention is a method of treating hearing defects comprising the step of administering to a mammal affected with said hearing defect a therapeutic effect amount of an agonist to COUP-TFI, wherein said agonist induces growth of the inner ear.

An additional embodiment of the present invention includes a method of treating balance defects in a mammal comprising the step of administering to a mammal affected with said balance defects therapeutic effective amount of an agonist to COUP-TFI, wherein said agonist induces growth in the inner ear.

Another embodiment of the present invention includes a method of inducing regeneration of bone formation in a mammal comprising the step of administering to said mammal a therapeutic effective amount of agonist of COUP-TFI, wherein said agonist induces bone formation.

Another embodiment of the present invention includes a method for

A further embodiment of the present invention includes a method of treating to the inner ear in a mammal comprising the step of administering to a mammal with an injured inner ear a therapeutic effective amount of an agonist to COUP-TFI, wherein said agonist induces growth or inhibits degeneration of the inner ear.

An additional embodiment of the present invention includes a method for enhancing the growth of the cochlear duct, scala tympani or sacculus in a mammal comprising the step of administering a therapeutic effective amount of an agonist to COUP-TFI, wherein said agonist induces growth.

Another embodiment of the present invention includes a method for treating neurodegenerative diseases in a mammal comprising the step of introducing by genetic therapy a COUP-TFI gene operatively linked to a promoter, wherein said COUP-TFI gene is inducible by the addition of an agonist and said expression of COUP-TFI enhances expression of COUP-TFI increasing the growth of neural and neuronal tissue.

Another embodiment of the present invention includes a method for treating bone diseases in a mammal comprising the step of introducing by genetic therapy a COUP-TFI gene operatively linked to a promoter, wherein said COUP-TFI gene is inducible by the addition of an agonist and said expression of COUP-TFI enhances expression of COUP-TFI increasing the growth of bone tissue.

An additional embodiment of the present invention includes a method for treating hearing and balance diseases in a mammal comprising the step of introducing by genetic therapy a COUP-TFI gene operatively linked to a promoter, wherein said COUP-TFI gene is inducible

It is demonstrated herein that mCOUP-TFI is essential for postnatal survival and is required for proper development of a subset of neurons in the peripheral nervous system. Evidence of this is provided herein, and includes experimental results showing that mutant embryos displayed a defective morphogenesis of the glossopharyngeal ganglion and nerve. In addition, inactivation of the mCOUP-TFI gene also resulted in defective axonal guidance.

Paradoxically, mCOUP-TFI is widely expressed, yet the phenotypes exhibited by the mutants were highly specific. This suggests that in certain regions, mCOUP-TFI might be compensated for by mCOUP-TFII. Despite this occasional compensatory effect, the fact that mCOUP-TFI knockout is lethal provides convincing evidence that mCOUP-TFI, beyond question, possesses a distinct physiological function, despite its extensively overlapping expression profile with mCOUP-TFII.

Example 1

The Method of Targeted Disruption of the mCOUP-TFI Gene

To assess the physiological function of COUP-TFs in vivo, disruption of one of the two mouse COUP-TF genes (mCOUP-TFI) by homologous recombination was undertaken.

A mouse 129Sv genomic library was screened with a mouse cDNA fragment containing the complete mCOUP-TFI open reading frame. Several of the positive clones contained all three exons of mCOUP-TFI gene. A 700 bp fragment containing 5' and primer untranslated region was excised with EcoRI/SacI and used as the 5' homologous arm in the gene targeting vector. The SacI site was destroyed during subsequent subclonings. A 6.5

was digested with SalI/XhoI and inserted in the same transcriptional direction as the mCOUP-TFI. The left arm. PGKneobpA and the right arm were assembled in pBluescript, released with XhoI/BamHI, and cloned into XhoI/BamHI of pSP72TK containing the herpes simplex virus thymidine kinase gene. The construct was linearized with XhoI before electroporation.

This technique is illustrated in Figure 1, which depicts targeted disruption of the mCOUP-TFI locus. The targeting strategy is shown at the top of the Figure. The mCOUP-TFI locus is shown on top with exons I-III in boxes. The targeting vector and targeting locus are shown below. The solid region represents open reading frame and open boxes represents the 5' and 3' untranslated regions. The hatched regions represent probes used in Southern blot analysis. The replacement vector includes a *PKG-neo* gene and a *tk* gene. The directions of transcription are indicated by arrows. The shaded box represents pBluescript vector. The left and rights arms are indicated by dashed lines. The SacI site at the right end of the left arm was destroyed during subcloning. The correctly recombined locus is shown at the bottom. Restriction sites are indicated above the lines. The following symbols are used: P, *Pst*I; S, *Sma*I; X, *Xba*I; R1, *Eco*RI; B, *Bam*HI; SL, *Sal*I.

Figure 2 shows the results of a Southern blot analysis on a representative litter from a mCOUP-TFI heterozygote intercross. Genomic DNA was digested with *Sac*I and hybridized with the 5' probe. A 1.6 kb and a 3.0 kb bands were generated from wildtype and mutant alleles, respectively. For the 3' probe, *Bam*HI was used. The 8 kb and 15 kb fragments were generated from the wildtype and mutant allele, respectively. Figure

mainly containing the 3' untranslated region of mCOUP-TFI was used as the probe. Note the absence of mCOUP-TFI mRNA in the homozygote. GAPDH was used as an internal control and is shown at the lower panel.

The ES cells were cultured and manipulated essentially as described by Robertson (1987). Briefly, 107 AB 1 ES cells were electroporated with 25 mg targeting construct in 0.9 ml PBS using a Bio-Rad Gene Pulser (500 μF, 230 V), and plated on either one or two 10 cm plates containing a monolayer of mitomycin C-treated fibroblast SNL76/7. Twenty-four hours later, the media was replaced with selection media containing 350 mg/ml G418 (GIBCO) and 0.2 mM FIAU, and cells were cultured for additional 9 days. Individual drug resistant colonies were picked and expanded in 96-well plates containing feeder cells.

For screening the ES cell colonies, the cells were trypsinized in situ and two thirds were frozen at -80^{0}C The remaining cells were replicated into a new 96-well plate without feeder cells and grown to confluency for DNA isolation. The DNA was isolated and digested within the 96-well plates as described previously . Southern blot analysis was carried out according to Church and Gilbert (1984). For genotyping mice and older embryos (E10.0 and older), genomic DNAs were isolated from tail biopsies or the yolk sac, respectively, following overnight proteinase K treatment and ethanol precipitation. The DNA was then subjected to Southern analysis or PCR. For younger embryos, the yolk sac was treated with proteinase K overnight, heated to 99^{0}C for 10 min, and used for PCR directly. To detect the wildtype allele, the 5, primer was 5'-CACGGACCAGGTGTCTCTG -3', located in a region of exon 2 that was deleted in the mutant allele. The 3' primer

5'- GCTATCAGGACATAGCGTTG -3', located at the 3' end of the PGKneo gene. PCR was performed according to the manufacture's instructions (Promega), with 1.0 mM $MgCl_2$, at the annealing temperature of 55°C.

The positive clones identified from the screening were thawed, expanded, and injected into embryonic day (E) 3.5 blastocysts derived from C57BL/6 females. The embryos were transferred into the uteri of pseudopregnant F I (CBA x C57BL/6) foster mothers.

Male chimeras with 80% - 100% agouti coat color were backcrossed to C57BL/6 females, and germline transmission was determined by the presence of agouti offspring. Heterozygotes were determined by Southern analysis and intercrossed to generate homozygotes.

Whole-mount immunohistochemistry with a monoclonal antibody (2H3) recognizing a 165 kD neurofilament protein was performed essentially as described (Wall et al. 1992). Briefly, embryos were collected in PBS, fixed in methanol:DMSO (4:1) overnight at 4°C. The embryos were then bleached in methanol:DMSO:30%H_2O_2 (4:1:1) for 4-5 hours at room temperature, rehydrated for 30 min each through 50% and 15% methanol, and finally PBS . Embryos were incubated twice in PBSMT (2% instant skim milk powder, 0. 1 % Triton X- 100 in PBS) for 1 hour at room temperature, then with primary antibody diluted in PBSMT at 4°C overnight. Embryos were washed in PBSMT twice at 4°C and 3 times at room temperature for 1 hour each, followed by an overnight incubation at 4°C with peroxidase-conjugated goat anti-mouse IgG diluted in PBSMT. The washes were similar to post primary antibody washes with an additional 20 min wash in PBT (0.2% BSA, 0.1% Triton X-100 in PBS) at room temperature. For the color reaction, embryos were incubated with 0.3 mg/ml diaminobenzidine

were then rinsed in PBT to stop the reaction, dehydrated through a methanol series: 30%, 50%, 80%, 100% for 30 - 60 min each, and cleared in benzyl alcohol:benzyl benzoate (1:2). Embryos were photographed on a Zeiss stemi 200° dissecting microscope.

Whole-mount in situ hybridization was performed according to Wilkinson (1992). A mCOUP-TFI cDNA clone containing the entire open reading frame was used as the template for transcribing digoxigenin labeled antisense probe. The same probe did not generate a hybridization signal in homozygote mCOUP-TFI mutant embryos. X-gal staining of the embryos was performed as described by Behringer et al. (1993). TUNEL assay was performed as described by Gavrieli et al. (1992). The embryos were stained with X-gal, then processed and embedded in paraffin. Neurons were counted from every 6th section of 7 mm thick sections. In the cases when ganglion IX and X were fused, the cells rostral to ganglion X were counted as cells for ganglion IX. Statistical significance was analyzed by Student's t-test.

A genomic clone, isolated from a mouse 129Sv genomic library, was used to construct the targeting vector. This clone contained the entire mCOUP-TFI gene which spans three exons. The N-terminal and the DNA-binding domain are in the first exon, whereas the ligand-binding domain is split into two exons. The targeting vector contains a 0.7 kb 5' homologous sequence and a 6.5 kb 3' homologous sequence flanking the neon cassette (Figure 1). A 4.0 kb genomic region including the N-terminus and the entire DNA-binding domain and two thirds of the ligand-binding domain will be deleted and replaced with the PGK-neobpA gene upon

The targeting vector was linearized, electroporated into the AB 1 ES cells and subjected to positive (G418) and negative (FIAU) selections. A total of 850 colonies were screened by Southern blot analysis. The restriction enzyme XbaI was used as the diagnostic enzyme and a 0.5 kb XbaI/EcoRI fragment just upstream of the 5' homologous sequence was used as probe. In the case of the wildtype mCOUP-TFI locus, a 8.0 kb fragment was generated while in the mutant allele, a 2.6 kb figment was produced since an extra XbaI site is introduced by the neor cassette. By employing this strategy, 13 positive clones were obtained. To ensure that proper recombination had occurred on both the 5' and 3' ends of the neor cassette, another diagnostic enzyme (SacI) was used and hybridized with the same probe. A 1.6 kb band and a 3.0 kb band were generated from the wildtype and mutant allele, respectively (Fig. 2A). In addition, a 1.0 kb PstI fragment which resides downstream of the 3' homologous sequence was used as a probe on BamHI digested samples. As expected, 8.0 and 15 kb bands were detected from wildtype and mutant alleles, respectively (Fig. 2A). Southern blots were stripped and reprobed with a fragment of the neor gene to ensure that there were no other insertions in the genome.

The ES cells carrying the mutated mCOUP-TFI locus were injected into C57BL/6 blastocysts. Two male chimeras with 100% agouti coat color generated from one ES clone were mated to C57BL/6 females. Germline transmission was obtained, and the resulting heterozygous mice appeared phenotypically normal and were fertile. Heterozygous mice were intercrossed to generate homozygotes. To ensure no normal mCOUP-TFI transcript was generated from the mutated allele, total RNA was isolated from E13.5 embryos from heterozygote

probe in Northern hybridization analysis. As shown in Fig. 2B, no mCOUP-TFI specific transcripts were detected in the homozygotes.

To define the physiological consequences of removing mCOUP-TFI in vivo, F1 heterozygous mice were intercrossed to generate homozygotes. Homozygotes were not recovered at the weaning stage (3 weeks after birth) during initial analysis of 100 F_2 offspring. However, a normal number of mutant pups were recovered at birth (newborn), in accordance with Mendelian inheritance, indicating that the homozygotes survive until birth. Within the first two days after birth, an unusual number of newborn deaths were noticed. Dead pups were genotyped and the vast majority of them were found to be homozygotes. A closer examination showed that homozygotes were indistinguishable from their wildtype or heterozygous litter mates at birth. However, within hours after birth, the homozygotes became easily identifiable due to the lack or minimal milk in their stomachs. Almost all homozygotes died between 8 and 36 hours after birth. After screening 901 offspring from heterozygote matings, two homozygotes were found to survive for three weeks, but they were very small, about ¼ the size of their litter mates, and appeared ataxic.

Example 2

Demonstration that COUP-TFI is Expressed in Premigratory and Migratory Neural Crest Cells

Selected aspects of the COUP-TFI expression pattern in mice, are uncovered in this Example. Similar, but more general studies have been performed in the chick, zebrafish, frog, and *Drosophila*, which show that the general patterns of expression in different species are, broadly speaking, similar.

and in many organs. Hence the expression pattern overlaps, yet is distinct from, that of COUP-TFII. In general though, COUP-TFI expression is higher in the CNS and lower in internal organs as compared with COUP-TFII.

Figure 8 shows the excessive cell death in mCOUP-TFI mutant embryos. mCOUP-TFI wildtype (Figures 8A-8D) and mutant (Figures 8E-8H) embryos at E9.5 were stained with X-gal, sectioned and TUNEL assay was performed. Every other section in the region of interest is presented. Sections at top are more dorsal than the sections at the bottom. Note that the left side of the mutant embryo shows much more pronounced cell death than in the wildtype. The following symbols are used: ot, otic vesicle; VII/VIII, facial and acoustic ganglia; X, vagus ganglion. Note that IX marks a position dorsal to ganglion IX proper which lies further ventral. The arrows indicate apoptotic cells.

The expression of mCOUP-TFI was evident at the 1-2 somite stage. This expression was increased at 4-6 somite stage in the neuroepithelial region corresponding to the presumptive rhombomeres (r) 1-3 in the hindbrain (Fig. 4A). At this stage, mCOUP-TFI expression was observed in a stripe extending ventrally from the dorsal tip of the middle of the neuroepithelial expression domain. The posterior boundary of the neuroepithelium corresponded to the posterior boundary of presumptive r3, as indicated by Krox-20 expression at the same stage suggesting the stripe originated from the presumptive r2. mCOUP-TFI transcripts could also be seen in the developing foregut. At the 10-12 somite stage, mCOUP-TFI was expressed at high levels in the neuroepithelium and the

ventrally from the presumptive r2 and r4 (Fig. 4B). The pattern and timing of the two stripes of mCOUP-TFI expression from r2 and r4 resemble that of the migrating neural crest cells. The neural crest cells (NCC) migrate through well defined pathways and occupy very characteristic positions. Sections of embryos stained by whole-mount in situ hybridization showed that some mCOUP-TFI expressing cells did occupy very characteristic positions of both premigratory and migratory NCC (Figures 5D, 5E). This conclusion was further substantiated by the similar expression patterns of mCOUP-TFI and several other known NCC markers, including AP-2 and cellular retinoic acid-binding protein I (Fig. 5A) at these stages. At E9.0, mCOUP-TFI was highly expressed in the entire hindbrain neuroepithelium and in the migrating NCC from r2, 4, and 6 to branchial arches 1, 2, and 3, respectively (Fig. 4C). The expression in the midbrain, forebrain, and other regions was similar to the pattern in later stages as reported previously.

Example 3

Demonstration of Defects in the Formation of the Glossopharyngeal Nerve in mCOUP-TFI Null Mutants

The results presented in this and following Examples clearly demonstrate that mCOUP-TFI is important for proper development of the peripheral nervous system and that mCOUP-TFI serves a vital function which is not shared by mCOUP-TFII.

Figure 3 depicts a whole-mount immunohistochemistry on wildtype and homozygote embryos using 2H3 anti-neurofilament antibody showing the progression of cranial ganglion formation (dorsal is to the left, ventral is to the right, scale bar, 100 μm).

of embryos. Arrows in Figures 3A and 3C point to the nerve projections between the IXth ganglion and the hindbrain; arrows in Figures 3B and 3D point to the absence of nerve projection between the IXth ganglion and the hindbrain. Arrowheads in Figures 3B and 3D also point to the connections between the IXth and the Xth ganglia. The following additional labels are used: V, trigeminal ganglion, VII, facial ganglion; IX, glossopharyngeal ganglion; X, vagus ganglion; XI, accessory ganglion; XII, hypoglossal nerve; and, ot, otic vesicle.

Anatomical analyses did not reveal any obvious structural defects in the cranio-facial region which would have compromised the suckling process. Thus, given the extensive neuronal expression of mCOUP-TFI, the nervous system of the null mutants was examined. A monoclonal antibody (2H3) raised against the 165 kD neurofilament protein was used in whole-mount immunohistochemical analyses to examine the peripheral nervous system. Thirty-seven embryos from embryonic day (E) 9.5 to E1 1.5 (25 somites to 45 somites) were stained, of which 15 were wildtype and 22 were mutant. The phenotype with the highest penetrance was an abnormality of the glossopharyngeal ganglion (IX) and its nerve. In wildtype embryos, ganglion IX and X are well separated with very few nerve fiber connections between the two ganglionic masses. Multiple axonal projections between the glossopharyngeal ganglion and the hindbrain are readily seen in wildtypes (arrows in Figures 3A, 3C); however, in mutants, ganglion IX appeared as an isolated mass of neurons (arrowhead in Figure 3B), or in other cases, a complete fusion or shunt of the axons away from the hindbrain and towards ganglion X (arrowhead in Fig. 3D). Indeed, embryos with a complete fusion of the IXth and Xth ganglia [illegible]

Out of 22 mutant embryos examined, only one embryo exhibited wildtype-like appearances for the glossopharyngeal nerve, presumably due to incomplete penetrance. The defect in the glossopharyngeal ganglion was commonly seen on a single side of a given embryo, with the other side appearing phenotypically wildtype. However, in the more severely affected mutants, both sides showed defects (one example is shown in Figures 9B, 9C, 9D, 9E, and 9F, which will be discussed later). No wildtype embryos examined appeared aberrant (Figures 3A, 3C, and Figure 9A). The observed defects in the glossopharyngeal nerve could result from several reasons. In order to study how the loss of mCOUP-TFI resulted in the defects, the expression of mCOUP-TFI in this and earlier developmental stages were examined in relation to the ontogeny of the IXth nerve.

The morphogenesis of the glossopharyngeal ganglion and its nerve is defective in mCOUP-TFI mutant embryos as shown by neurofilament staining. The glossopharyngeal nerve is the nerve of the 3rd pharyngeal arch. It supplies both sensory and motor innervation to the pharynx and root of the tongue. It also innervates the middle ear and soft palate. Together with the vagus nerve (the Xth cranial nerve), the IXth nerve registers and regulates blood pressure and pulse rate. The cell bodies of the sensory and taste fibers reside in the IXth ganglion and the fibers terminate in the nucleus solitarius in the hindbrain. The motor fibers originate from the cranial part of nucleus ambigius and the secretory fibers stem from the inferior salivary nucleus in the hindbrain. These fibers pass through the ganglion and terminate on the target organs. Thus, proper connection between the ganglion and the appropriate nuclei in the brainstem is

the Xth ganglion. Indeed, there were no connections to the hindbrain through its normal route. Therefore, the function of the glossopharyngeal nerve appears severely compromised. Since the mutants die from apparent starvation and dehydration, they probably have difficulty in obtaining exogenous nutrients which are critical for their survival. Among the targets of the glossopharyngeal nerve, the innervation to the pharynx and the root of the tongue, might be most important regarding the lethal phenotype of mCOUP-TFI mutants, since proper control of the tongue and the pharynx is required for proper suckling and swallowing behavior. In fact, when tested by putting a drop of milk around their mouth, wildtype or heterozygotes could easily drink and swallow 30-50 μl of milk. In contrast, the mutants displayed abnormal throat movements after taking in the milk and soon the milk was expelled from the nasal cavity, suggesting that the mutant pups did exhibit difficulty in swallowing. Yet, since in most cases, the defect in the IXth ganglion was only observed at one side, this was probably not the sole contributor to the lethality of the mCOUP-TFI mutant pups. Indeed, since the IXth ganglion was often fused with the Xth ganglion in the mutant embryos. It is possible that the function of the Xth nerve is compromised as well.

The ganglion of the glossopharyngeal nerve contains two components: the superior and the inferior (petrosal) ganglion. In the superior ganglion, both the neurons and the non-neuronal cells are derived from the neural crest cells of r6. In the inferior ganglion, the neurons originate from the second epibranchial placode, while all the non-neuronal cells are derived from neural crest cells of r6. Since mCOUP-TFI is expressed

morphology of the IXth ganglion in the mutant embryos supports this hypothesis. However, there is no easy way to test this hypothesis since there is no well characterized molecular marker that only expresses in the superior ganglion. The defect would range from improper neural crest cell fate specification, migration, to differentiation. Analysis using various rhombomere specific and migrating neural crest markers did not reveal any consistent difference between mutant and control embryos in terms of the expression pattern and profile, especially in the r6 region and its associated neural crest cells. This demonstrates that the NCC from r6 were appropriately fated and migrated towards arch 3 in mCOUP-TFI mutant embryos. This conclusion was consistent with the observation that the muscular and skeletal derivatives of the 3rd pharyngeal arch were apparently normal in mCOUP-TFI mutant pups. The majority of the NCC from r6 migrate into pharyngeal arch 3 which later differentiates into the greater horn and lower portion of the body of the hyoid bone and the stylopharyngeus muscle. More importantly, the NCC marker gene expression implied that the NCC destined for the superior component of the IXth ganglion were fated and migrated properly. However, the number of neurons in the IXth ganglia of the mutant embryos was reduced by 40% when compared to that in the wildtype embryos at E10.5. This suggests that either the NCC for the IXth ganglia in mutants are defective in proper differentiation or that the neuron loss in the mutant ganglia is not of neural crest origin.

To clarify the possibilities, the extent of cell death in these regions was analyzed. There is a limited amount of cell death in specific regions during normal embryonic development. The best known example is the

Most of the apoptotic cells in other regions are neural crest in origin. On the other hand, the life or death decision of the neural crest cells is influenced by environment and position. For example, the neural crest cells generated in r3 and r5 do not emigrate, instead, they undergo apoptosis. Yet when r3 or r5 is explanted into culture, the neural crest cells migrate out perfectly because now they are free from the influence of the even number rhombomeres. Cell death has been detected along the migratory routes of neural crest cells, including regions of the developing VII/VIIIth ganglia and the IXth ganglion. At late E9.5 embryos, a few apoptotic cells were observed in a region just dorsal to the developing IXth ganglion in wildtype or heterozygous embryos. In contrast, much more pronounced cell death was observed in a similar region of the mutant embryos. The apoptotic cells occupied the same region as the neural crest cells for ganglion IX. Thus, more than likely, at least some of the apoptotic cells are neural crest in origin which in turn shows that the neuron loss is in the superior component of the IX ganglion. Collectively, this evidence proves that the excessive precursor cell death results in a decrease of neurons in the glossopharyngeal ganglia of mCOUP-TFI mutant embryos, and that the defect is in the superior component.

Since neural crest cells contribute to all the non-neuronal cells in both superior and inferior ganglion and since non-neuronal cells are present in the IXth ganglia of the mutant embryos, it is clear that not all the neural crest cells for the IXth ganglion undergo apoptosis. It is not known whether the neurons selectively undergo apoptosis. Yet, it is possible that there are also less non-neuronal cells in the mutant IXth ganglion than in the wild-type

Although the number of neurons in the Xth ganglia of the mutant embryos did not differ significantly from the wildtype or heterozygous embryos, it is possible that the formation of the Xth ganglion is slightly compromised.

Neural crest cell death during normal embryonic development could either be due to response to an over-production of the neural crest cells or it could contribute to patterning the neural crest migration pathway. The excess death observed around the mutant IXth ganglion is a consequence of the lost of function of the mCOUP-TFI gene. Thus, this group of cells probably did not receive or could not respond to appropriate signal(s) for differentiation and switched to programmed cell death or apoptosis instead. Besides the IXth ganglion, the Vth, VII/VIIIth, and Xth ganglia also have neural crest contribution. Yet, obvious defects were only observed on the IXth ganglion. Thus it appears that the formation of the other ganglia does not require mCOUP-TFI function. Alternatively, the lack of mCOUP-TFI function in those regions is compensated by other genes such as mCOUP-TFII which is expressed similarly as mCOUP-TFI although in a less restricted manner.

Example 4

Demonstration of Reduced Number of Neurons in the IXth Ganglion in mCOUP-TFI Null Mutants

Even though the NCC migrate appropriately, it is important to know whether the correct *number* of neurons exist at the IXth ganglionic position.

Figure 7 shows the development progression of the formation of the IXth ganglion as visualized by X-gal staining. mCOUP-TFI wildtype (A, C) and mutant (Figures 7B and 7D) embryos at E9.5 (Figures 7A and 7B)

[illegible] ganglion IX.

glossopharyngeal ganglion; X, vagus ganglion. The arrow indicates the fusion between the IXth and the Xth ganglia in the mutant embryo.

The ganglionic mass at the IXth ganglion was fractionally reduced in the more dorsal aspect of the ganglion. In order to visualize the ganglionic cells more easily, mCOUP-TFI mutants were crossed into the BETA2-LacZ background. BETA2/NeuroD is a basic helix-loop-helix transcription factor. A lacZ gene with a nuclear localization signal was placed in-frame with the BETA2 open-reading-frame when the BETA2 knockout mice were generated. BETA2 is expressed early in developing cranial ganglia as visualized by X-gal staining. The first few differentiating neurons stained blue were observed at the 17 somite stage in the trigeminal ganglion and subsequently the blue neurons were observed in all the differentiating cranial ganglia. Mice heterozygous for the BETA2 gene have no detectable defects. The BETA2 gene expression was thus used as a marker for ganglionic cell bodies in our study. The defect in the mutant IXth ganglion was fully manifested at E10.5 (Figure 7C, 7D), the number of neurons in both wildtype and mutant IXth ganglia were counted. Indeed, the number of neurons in ganglia IX was significantly lower (about 40%) in the mutants than in the wildtypes or heterozygotes. As a control, neurons in the Xth ganglia of the same embryos were counted and the numbers were not significantly different between the mutant and the wildtype or heterozygous embryos. This shows that some precursor cells of ganglion IX must have prematurely died, changed fate or migrated to a different position.

Example 5

Demonstration that Segmentation and Identify of the Hindbrain and NCC Migration is Largely Unchanged in COUP-TFI Null Mutants

In light of the diverse expression pattern of mCOUP-TFI in the neuroepithelium, premigratory and migratory NCC, the observed defects in the glossopharyngeal nerve may be a result of loss of mCOUP-TFI function in any or all of these regions. Thus, the possibilities were examined in detail.

Figure 4 depicts the Expression of mCOUP-TFI. A-C, whole-mount in situ hybridization of mCOUP-TFI in E8.0 (Figure 4A), 8.5 (Figure 4B), and 9.0 (Figure 4C) embryos, respectively. Figures 4D-4F show sections of a whole-mount stained E8.5 embryo. The following symbols are used: a1, a2, and a3, branchial arch 1, 2, and 3, respectively; drg, dorsal root ganglia; fb, forebrain; h, heart; hb, hindbrain; hf, head fold; fg, foregut; mb, midbrain, ot, otic vesicle; pmnc, premigratory neural crest cells; r2c, r4c, neural crest cells from r2, r4 respectively; sm, somite. The arrows in Figure 4D point to premigratory neural crest cells (NCC); arrows in Figures 4E and 4F indicate migrating NCC that are mCOUP-TFI positive.

Figure 9 show whole-mount analysis of axonal projections. Multiple defects in cranial nerve projections are detected in severely affected embryos. A, wildtype E10.5 embryo. The right (Figure 9B) and the left (Figure 9C) sides of a E10.5 mutant embryo. Figure 9D and 9F show enlargement of cranial nerve IX-XII region from Figures 9B and 9C. Note the isolated ganglionic mass (asterisk in Figure 9D) at the position of the IXth ganglion, the abnormal projection of a single nerve fiber towards the hindbrain (Figure 9D) and the abnormal axonal bundles (asterisk in Figures 9D

enlargement of the oculomotor nerve (III) region in B and C. Note also the abnormal projection of nerve III on the left side (asterisk in H). The following symbols are used: III, oculomotor nerve. V, trigeminal ganglion; VII, facial ganglion; IX, glossopharyngeal ganglion; X, vagus ganglion; XI, accessory ganglion; XII, hypoglossal nerve.

Figure 5 shows the expression of rhombomere specific genes in mCOUP-TFI mutants. Whole-mount *in situ* hybridization was performed on wildtype (5A-5C) or mutant embryos (Figures 5D-5F) with rhombomere specific markers. Note that the expression is unchanged for mutants. Figures 5A and 5D: Krox-20 expression in r3, r5 of E8.5 embryos. Figures 5B and 5E: Hoxb-1 expression in r4 of E9.0 embryos. Figures 5C and 5F show that CRABP I expression is strong in R4-6, but weak in r2 for E9.5 embryos.

Figure 6 depicts an analysis of cranial NCC migration in mCOUP-TFI mutants. Wildtype (Figures 6A, 6C and 6E) or mutant embryos (Figures 6B, 6D and 6F) were hybridized with CRABP I antisense probe to examine the migration of the neural crest cells. Whole-mount *in situ* hybridization on E9.0 (Figure 6A, 6B) and E9.5 (6C through 6F) embryos. Figures 6E and 6F show *in situ* hybridization with CRABP I antisense probe on sections of late E9.5 embryos. The following symbols are used: c9-10, neural crest cells for ganglia IX and X; fn, frontal nasal mesenchyme; r, rhombomere; r2c, r4c, r6c, neural crest cells from r2, r4, r6 respectively; ot, otic vesicle; 4, 5, and 6, r4, r5, and r6 respectively.

mCOUP-TFI is expressed in NCC since part of the IXth ganglion is derived front these specialized cells. The glossopharyngeal ganglion has two components: the

mCOUP-TFI is expressed in the NCC strongly suggests that the superior component of the IXth ganglion is defective in mutant embryos; however, in the mouse, unlike the chick, the superior and inferior components of the IXth ganglion are anatomically indistinct at these stages. In addition, since there is no known molecular markers that could distinguish the superior component from the inferior one in the ganglion, analyses must be performed at earlier embryonic stages before the NCC reach their final destination. Neural crest cells are prepatterned in the hindbrain and carry the identity of the rhombomere from which they emigrate. The identities of the rhombomeres are established at molecular levels via a combinatorial expression of a number of genes at segmentally restricted patterns during hindbrain development. In order to examine whether the segmentation and identities of the rhombomeres were retained in the mCOUP-TFI mutant embryos, several genes that are expressed in a segmentally restricted fashion in the developing hindbrain were used as markers for the respective rhombomeres. The expression of Krox-20 in mCOUP-TFI mutant embryos (Figure 5D) f lowed the same profile as in wildtype embryos (Figure 5A), where Krox-20 expression was first detectable in the future r3 around E8.0 and the second stripe appeared in the future r5 around E8.5. Subsequently, the expression in r3 decreased at E9.5 as previously defined. Hoxb-1 is expressed at a high levels in the neural tube at E8.0, extending from the posterior end of the embryo into the hindbrain with a sharp anterior boundary corresponding to the presumptive r3/r4 boundary. By E8.5, the expression domain in the neural tube retracts posteriorly except for the expression in r4 which persists until later stages. This dynamic expression of

hindbrain neuroepithelium of E8.5 to 9.5 embryos, CRABP I is highly expressed in r4, r5, and r6 and much lower in r2. Similar expression patterns were observed in both wildtype and mutant embryos (Figures 5C and 5F). From these results it is concluded that all the rhombomeres have segmented appropriately and that their identities, as determined by rhombomere specific molecular markers, are largely unchanged in the mCOUP-TFI mutant embryos.

Next, the migration of the NCC was examined. CRABP I is also an established marker for the migrating NCC. In E9.0-9.5 embryos, it is intensely expressed in the migrating NCC from r4, r6 and weaker in those from r2 (Figure 6A, 6B). The expression pattern of CRABP I in mCOUP-TFI mutant embryos is similar to that in wildtype embryos (Figures 6D, 6E). This result suggests that the migration of the cranial NCC is appropriate in mCOUP-TFI mutant embryos. To examine the NCC for the IXth ganglion, in situ hybridization was performed on coronal sections of E9.5 embryos using CRABP I antisense RNA as a probe. In both wildtype and mutant embryos, the neural crest cell population for the IXth and the Xth ganglia were appropriately observed caudal to the otic vesicle (Figures 6C and 6F). This study suggests that the neural crest cells that would form the superior component of the glossopharyngeal ganglion migrated properly.

Example 6

Demonstration of Pronounced Cell Death in a Region Dorsal to the IXth Ganglion in mCOUP-TFI Null Mutant Embryos

Some cell death occurs during normal embryonic development at early stages and patterns of the cell death correspond to routes of neural crest migration.

stained late E9.5 embryos (Figures 8A, 8B). Consistent with previous reports, limited cell death was observed in regions of developing cranial nerve migration routes including those of the IXth nerve in wildtype or heterozygous embryos (Figures 8A-8D). In contrast, similarly staged mutant embryos showed much more pronounced cell death in the corresponding region just dorsal to the IXth ganglion (Figures 8E-8H). The region of cell death corresponded to the expression domain of CRABP I and to the known migratory route of NCC for the IXth ganglion (compared to Fig. 7C, 7F), suggesting that at least some of the apoptotic cells in the mutant embryos might be of neural crest origin. In most cases, the excess cell death was only seen on one side of the mutant embryos. This correlates well with the observation that the defective IXth ganglion was commonly seen on one side of a given mutant embryo by neurofilament staining. Collectively, these data show that the excess cell death during the migratory phase of the NCC result in a decrease in differentiating neurons in the mutant ganglion IX. This in turn shows that the superior component of the glossopharyngeal ganglion is most likely compromised.

Example 7

Demonstration of Defects in Axonal Guidance in mCOUP-TFI Null Mutants

Besides being defective in the formation of the superior ganglion, the IXth ganglion in mutants formed no correct connections with the hindbrain (Figures 9E and 9F). Instead, axons fasciculated with cranial nerve X in some cases. Thus, this is consistent with an axonal guidance defect. In fact, this is a more general problem in mCOUP-TFI mutants. In addition to the defect in the glossopharyngeal nerve, abnormalities in other

display aberrant nerve fibers running down the caudal

part of the hindbrain on both sides of the embryo (see asterisk in Figure 9E, 9F). The origin of the nerve fibers was not clear. In addition, the oculomotor nerve (III) at the left side of the embryo (Figure 9H) appeared as a shortened and broadened bundle when compared to the right side or wildtype embryos (Figure 9G).

In addition to the aberrant projections, mutant embryos also displayed aberrant arborization at later developmental stages. When older embryos (E11-5 to E13-5) were stained with the 2H3 antibody, it was clear that the extent of arborization or branching of the axonal trees was affected in about half of the mutant embryos at the facial and cervical plexus regions. The cervical plexus is formed by the first four spinal nerves. In E11.5 wildtype embryos, the nerve fibers were extensively arborized (Figure 9A), whereas in the mutants, the primary axons appeared thicker and there were less secondary branchings and much less tertiary or higher order branchings (Fig. 9B). A similar defect was also observed in the ophthalmic branch of the trigeminal nerve (Fig. 9C, 9D), where there were fewer secondary as well as high order branchings. The arborization in other regions of the mutant embryos appeared relatively normal. These results clearly suggest that mCOUP-TFI is required for normal axonal guidance and arborization in a subset of neurons in the peripheral nervous system.

Figure 10 shows that mCOUP-TFI mutant embryos display reduced arborization of axonal trees. Whole-mount immunohistochemistry of wildtype (Figure 10A and 10C) and mutant E11.5 fetuses (Figure 10B and Figure 10D) using 2H3 anti-neurofilament antibody. Figure 10A and Figure 10B show sagittal views at the level of

Figures 10C and 10D depict higher magnification of the ophthalmic branch of the trigeminal nerve. Arrowheads point to the corresponding branching points in the wildtype and mutant embryos. Note also the extensive branchings in the wildtype, and the diminished extent of arborization in the bracketed region.

In developing embryos, nerve fibers have to navigate for long distances before reaching their targets. The pathfinding process has high fidelity due to the guidance molecules presented in the surrounding environment which are sensed by the growth cones. There are several kinds of guidance cues, including long range (diffusible molecules) and short range (contact mediated), both could be positive or negative. Normally, multiple guidance cues in the surrounding environment are presented to a given growth cone. The net outcome is determined by the balance between different forces. The nerve fibers in the cervical plexus and facial regions of many mCOUP-TFI null mutants were much less arborized than their wildtype or heterozygous litter mates and can be assigned to defects in axonal guidance. Axonal branches could either be formed by collateral initiation of secondary growth cones along the axon shaft or by bifurcation of the primary growth cones. This process involves initiation, stabilization, and outgrow of the secondary growth cones. Many transient branches are formed, but only few are stabilized. The higher order branches are formed in a similar way. Both the initiation and subsequent guidance of the secondary growth cones are believed to be governed by the same forces that guide the primary growth cones.

This abnormal axonal guidance in mCOUP-TFI mutant embryos could be a result of a loss of mCOUP-TFI in the

component affects the proper development of the remaining ganglionic cells. Conversely, the apoptotic cells in the mutants included some cells that would themselves present guidance cues for the proper axonal projection to the hindbrain. In addition, the aberrant arborization in the cervical plexus is probably a defect in the dorsal root ganglionic neurons since they are also derivatives of the NCC. The other obvious possibility is that the expression of a guidance molecule(s) is altered due to lack of mCOUP-TFI function. Most of the affected nerves fail to do not grow or arborize sufficiently, yet severely affected mutants have aberrant fibers running down the hindbrain which implies an overgrowth of the axons. Whether the defects in axonal guidance in the various regions are directly caused by changes in a single guidance molecule or by multiple molecules, is not certain. Even if the expression of a single guidance molecule is altered in mCOUP-TFI mutants, different phenotypes could be manifest because it is the collective information from attractive and repulsive guidance cues in the environment that determine the action of a growth cone. Furthermore, one guidance cue could be positive for some axons yet negative for others.

Example 8

Demonstration of Fusion of the Basioccipital and Exoccipital Bones in mCOUP-TFI Null Mutants

Protocols were established to determine additional physiological roles of COUP-TFI. As before, this involved generating mCOUP-TFI null mutant mice using homologous recombinant technology, and performing histopathological examinations on the mice after death. In this Example, the development of the bones forming the skull in COUP-TFI null mutants was closely

newborn mutants appeared highly dehydrated, lacked milk in their stomachs, and contained air in their intestines. After death, the mice skulls were examined histopathologically. As evidenced by Figure 11, major observable defects found in the null mutants are the fusion of the two exoccipital with the basioccipital bones of the skull, and malformation in the cartilage between the basioccipital and exoccipital bones.

Figure 11 is comprised of six different photographs, labeled as Figures 11A-11F. Figures 11A-11D show skulls of newborn mice, and 11E-11F show skulls of 17-day fetal mice. Figures 11A-11D show whole-mount views of alizarin red (mineralized bone) and alcian blue (cartilage) stained skulls of newborns; Figures 11E and 11F show 17-day fetuses of wildtype (A); Figures 11C and 11E show heterozygotes; Figures 11B, 11D and 11F show COUP-TFI mutants. Note the double (11B) and single (11D) fusion of the basioccipital (bo) and exoccipital (ex) bones in the mutants. Figure 11 also clearly shows the malformation in the cartilage (extra hole, marked with arrowhead in 11F) between the basioccipital and exoccipital bones in the 17-day mutant skull.

Example 9

Demonstration of Defects in the Cervical Vertebrae in COUP-TFI Null Mutants

Protocols were established to determine still additional physiological roles of COUP-TFI. As before, this involved generating COUP-TFI null mutant mice using homologous recombinant technology, and performing histopathological examinations on the mice after death. In this Example, the development of the cervical vertebrae in COUP-TFI null mutants was closely examined.

in their stomachs, and contained air in their intestines. After death, the mice cervical vertebrae were examined histopathologically. As evidenced by Figure 12, the most striking features found in the null mutants are the lack of mineralization in the traberculi anterior, and bifurcation and duplication of the neural arches.

More specifically, Figure 12 shows whole-mount views of cervical vertebrae in wildtype (12A and 12B) and COUP-TFI null mutants (12C and 12D). The individual vertebrae are denoted as C1-C7, according to the legend between 12A and 12B. Figures 12A and 12C are frontal views and 12B and 12D are dorsal views. Figure 12C clearly shows the lack of mineralization in the traberculi anterior at C6 (as evidenced by the arrow). Figure 12D shows bifurcation and duplication of the neural arches in C2 (shown by the arrow).

Example 10

Demonstration of Defects in the Inner Ear in COUP-TFI Null Mutants

Protocols were established to determine additional physiological roles of COUP-TFI. As before, this involved generating COUP-TFI null mutant mice using homologous recombinant technology, and performing histopathological examinations on the mice after death. In this Example, the development of the structures forming the inner ear in COUP-TFI null mutants was closely examined.

As before, the COUP-TF null mutants died perinatally between 8 and 36 hours post-birth. The newborn mutants appeared highly dehydrated, lacked milk in their stomachs, and contained air in their intestines. After death, the mice inner ears were examined histopathologically. As evidenced by Figures

mutants, the widened chamber of the scala tympani, and defects in the formation of the sacculus.

More specifically, Figure 13 is a whole-mount view showing the inner ear of wildtype (+/+) in Figure 13A, and COUP-TFI mutant (-/-) in Figure 13C. As evidenced by Figures 13A (top left) and 13C (top left), the cochlea from the wildtype (right) has approximately 2.5 turns of cochlear duct (black line is drawn through the middle of the cochlear duct to better illustrate the number of turns). Yet the cochlea from the null mutant has less than 2 turns.

Figures 13B and 13D (lower right and left) are haematoxylin- and eosin-stained sagittal views through the mid-modiolar plane of wildtype (13B) and mutant (13D) cochlea, showing the basal coil (BC) median coil (MC), and apical coil (AC) of the cochlear ducts. Most prominently, the chamber of the scala tympani (ScT) in the null mutant (right) is much wider than in the wildtype.

Further experiments were performed to investigate additional functions of COUP-TFI related to the inner ear. Results from these experiments are shown in Figure 14. In Figure 14 wildtypes are shown along the top row, in Figures 14A-14D. Mutants are shown along the bottom row in Figures 14E-14H. Together, these eight snapshots are whole-mount and sectional views of alizarin red- (mineralized bone) and alcian blue- (cartilage) stained inner ears showing defects in formation of the sacculus. Figure 14G clearly shows the absence of a pink- or black-stained sacculus membrane in mutants. Yet a comparison of Figures 14D (wildtype) and 14H (mutant) illustrate that the middle ear bones of mutants appear unaffected. The following symbols are used: U, utriculus; S, sacculus; SC,

Example 11

Identification of Ligand Agonists and Antagonists of COUP-TFI

Classical steroid hormone receptors (androgen, estrogen, glucocorticoid and progesterone) have been extensively characterized in terms of their ligands. Numerous ligand agonists and antagonists have been identified to regulate physiological processes as diverse as inflammation to pregnancy to abortion to cancers. An important aspect of the present invention is a method to determine specific ligand agonist(s) and antagonists for the COUP-TFI. These are important tools for regulation—to activate or repress—COUP-TFI specific target genes. The ligands for other members of the superfamily comprising COUP-TFI include steroids, retinoids, thyroid hormones, vitamin D, oxysterols, prostaglandins or any lipid-soluble compounds. It is believed that the ligands for orphan receptors of this family are small hydrophobic or amphipatic molecules. COUP-TFI is a typical member of the superfamily of ligand-activated nuclear transcription factors; it has a typical putative-ligand binding domain (LBD). This LBD is highly conserved within the superfamily and between different members as diverse as from human to *Drosophila* within the COUP-TF subfamily, from which one can infer the existence of a ligand for the COUP-TF subfamily. The LBD has a pocket structure capable of binding a synthetic molecule as a ligand and can be regulated. Thus, a shotgun approach is used to identify the ligand of a given receptor in which an extensive battery of purified compounds known to affect axon guidance or bone differentiation are screened. Conversely, classical biochemical purification and concentration of extracts is used in

is prepared to test its ability to activate the receptor in cotransfection assays. This technique is described in: Heyman R. A., Mangelsdorf D. J., Dyck J. A., Stein R. B., Eichele G., Evans R. M., and Thaller C., *9-cis retinoic acid is a high affinity ligand for the retinoid X receptor*. Cell, 68, 397-406 (1992); Forman B. M., Tontonoz P., Chen J., Brun R. P., Spiegelman B. M., and Evans R. M., *15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma*. Cell, 83, 803-812 (1995). Using this technique, ligands have been identified for the retinoid (RAR and RXR), LXR and peroxisome-proliferator activated receptors (PPAR) which include retinoid and cholesterol metabolites, and prostaglandins which have diverse biological functions including teratogenic and anti-diabetic effects.

In an initial screen for ligands, a chimeric receptor is created in which the ligand-binding domain of COUP-TFI is fused to the DNA-binding domain of the yeast transcription factor GAL4. This technique is described in: Willy P. J., Umesono K., Ong E. S., Evans R. M., Heyman R. A., and Mangelsdorf D. J., *LXR, a nuclear receptor that defines a distinct retinoid response pathway*. Genes & Development, 9, 1033-1045 (1995). The resultant GAL4-COUP-TFI expression plasmid is then cotransfected together with a GAL4-responsive luciferase reporter plasmid into CV-1 cells and challenged with concentrates from several tissue sources. A significant (greater than twofold) induction of luciferase activity indicates the presence of a molecule in the extract capable of controlling the GAL4-COUP-TFI chimeric protein. The lipid extract activity is then fractionated on reverse-phase high-pressure liquid chromatography (HPLC) and the

An alternate approach to identification of COUP-TFI ligands is the use of an *in vitro* ligand-binding assay where the binding of a ligand to a steroid receptor induces a well defined and easily detected conformational change. This technique is fully described in U.S. Pat. Appl. Ser. No. 08/448,270, Tsai et al., *Method of Identifying Hormone Agonists*. The conformational change(s) is located within the ligand-binding domain and can be detected by partial proteolytic digestion or by a fluorescence change. This assay is used to detect binding of ligands to COUP-TFI, and forms the basis for identifying unknown compounds. Then, using COUP-TFI as an affinity matrix to trap the ligand, based on its characteristic high-affinity and specificity of binding, the ligand is further purified and identified by HPLC and GC/MS methods as above. The functional activity of this COUP-TFI ligand is then analyzed in chimeric receptor-reporter cotransfection assays and other axon guidance- or bone differentiation-specific assays.

In vitro analysis of conformational changes has several advantages over the classical *in vivo* cotransfection assay. It is an *in vitro* assay that directly detects ligand-receptor binding. *In vivo* assays cannot distinguish between ligand-dependent and -independent activation processes. The amount of potential ligand that is required for the *in vitro* assay is low because of the small reaction volume (5 μl) in comparison to the volumes of culture media and amounts of ligands required for *in vivo* assays. In addition, if the ligand is a hydrophilic compound, it may not be able to cross the cell membrane and enter the cell without an active transport mechanism. Furthermore, the assayed ligand may be toxic to the

not determined by the above method—i.e., whether it represses or activate its target gene. Therefore, at least two assay systems are devised to identify COUP-TFI ligands.

Example 12

Identification of Expression Regulators of COUP-TFI

In addition, a particular therapy may also exploit the known expression regulators of COUP-TF, either as an alternative to, or in addition to, the ligand agonist/antagonist. For instance, all *trans*- and *cis*-retinoic acids have been shown to induce expression of COUP-TFI. (See, Jonk, L.J.C., *et al.*, *Cloning and Expression During Development of Three Murine Members of the COUP Family of Nuclear Receptors*, 47 Mec. Dev. 81 (1994).) In addition, both RAR and RXR specific ligands are required for COUP-TFI gene expression.

Example 13

COUP-TFI is important for the formation of cortical layers

COUP-TFI is highly expressed in the central nervous system (CNS). The effect of mutations of COUP-TFI in mice on the formation of the CNS was determined. Upon examination of mutant mice at P0 (the day after birth), it was observed that the cortex of P0 COUP-TFI null mice is less differentiated (Figure 15). First, the cortex in the mutant is thinner than that in the wildtype mice, especially at the dorsal-lateral regions. In addition, the cortical subplate (sp) is hardly visible in the mutant. In order to support the conclusion that the subplate is defective, Gap43, an axon growth cone marker, was used to label 17.5 day embryos. As shown in Figure 16, data from both bright- and dark field indicate that the subplate is indeed

Since the subplate is the waiting station for the migrating neurons to the cortical plate, it is possible that formation of the cortical plate at later stages of animal life are affected. Analyses of 3 week-old mice (1-2% of animals survive more than 2 days), showed that cortical layers are poorly differentiated (Figure 17). Layer IV, which has small nuclei, is missing and layers II/III, as well as V/VI, are reduced. These cortical layers are important for receiving (IV) and sending out (II/III and V/VI) neuronal signals. These results, taken together, indicate that COUP-TFI plays an important role in the development of cerebral cortex and thus the proper communication of different part of brain. In addition, these mice show an impairment of communication between the right and left hemispheres due to a failure of axons of the curpus collosum and the hippocampal commissures to cross the midline, and a reduction of the size of the hippocampus and dentate gyrus. Since the hippocampus and dentate gyrus are critical centers for learning and memory, COUP-TFI is also important for learning and memory.

Example 14

COUP-TFI is important for the myelination of axons

In addition to the cortical layer defect of COUP-TFI mutant, the axonal myelination using luxol fast blue, which only stains myelinated axon was also examined. As shown in Figure 18, myelination of axons in white matter and striatum areas is much reduced. This result is further supported by the result shown in Figure 19; myelin basic protein staining of these two regions is drastically reduced. This is expected since without proper myelination, myelin expressed in the glia, cells will be degraded through lysosome pathway.

It was reported that Tst-1/Oct6/SCIP, a POU domain transcription factor, is necessary for the myelination of axon. In order to see whether Tst-1 expression was affected in the COUP-TFI knock out mice, *in situ* hybridization using Tst-1 probe was carried out. As shown in Figure 20, Tst-1 expression is missing in the cortex of mutant mice. This result clearly suggests that Tst-1 is a target gene of COUP-TFI and myelination defect of COUP-TFI mutant is due to the lack of Tst-1 expression. Since proper myelination of axons is important for axons to transmit neuronal signal, defect in the axon myelination will result in diseases such as multiple sclerosis.

Example 15

Novel Therapies

The Examples recited above clearly demonstrate, for the first time, the independent physiological role of COUP-TFI. Having determined the structure of COUP-TFI, its biochemical characteristics, expression pattern, physiological role, and regulation of its expression, Applicants submit herein that the findings from these experimental protocols can be exploited to craft novel therapies for the treatment of disorders whose etiological source is COUP-TFI, referred to as "COUP-TFI directed therapies."

More particularly, novel therapies can be directed at various points in the mechanism by which COUP-TF exerts its physiological effects. As discussed above, numerous ligands (both agonists and antagonists) have been identified for members of the superfamily of which COUP-TFI is a member. According to the standard model, a ligand agonist binds to the COUP-TFI, which induces a conformational change in the COUP-TFI, which then

some instances, it may suppress its expression). An antagonist also binds to COUP-TFI, but does not induce the proper conformation change to allow the complex to bind to the response element and trigger the proper transcriptional event. Therefore, ligand agonists of COUP-TFI are important therapeutic agents, as are antagonists, which are useful to modulate the effect of agonists.

Similarly, the therapy may also be directed at regulating the expression of COUP-TF, either as an alternative to, or in addition to, the ligand agonist/antagonist. For instance, all *trans*- and *cis*-retinoic acids have been shown to induce expression of COUP-TFI. (See, Jonk, L.J.C., *et al.*, *Cloning and Expression During Development of Three Murine Members of the COUP Family of Nuclear Receptors*, 47 Mec. Dev. 81 (1994).) Third, transfection of the COUP-TFI gene into the mammal is also a therapeutic option, as is direct administration of COUP-TFI. Finally, the subject could be administered COUP-TFI directly. Each of these techniques could, of course, be combined, or used singly. Support for each of these techniques is provided above.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art readily appreciates that the invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned

learning and memory defects with agonists to COUP-TFI, the methods of screening for agonists and antagonists to COUP-TFI, animal models, compounds, pharmaceutical compositions, treatments, methods, procedures and techniques described herein are presently representative of the preferred embodiments and are intended to be exemplary and are not intended as limitations of the scope. Changes therein and other uses will occur those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the pending claims.

WHAT IS CLAIMED IS:

CLAIMS

1. A method for treating a neurodegenerative disease in a mammal comprising administering to a mammal affected with said neurodegenerative disease a therapeutic effective amount of an agonist of COUP-TFI, wherein said agonist induces growth of neurological tissue.

2. The method of claim 1, wherein the neurodegenerative disorder is selected from the group consisting of Alzheimer's Disease, Huntington's Disease, seizure, Parkinson Disease, stroke, multiple sclerosis, and learning and memory defects.

3. A method of enhancing nerve regeneration in a mammal comprising the step of administering to a mammal an effective amount of an agonist of COUP-TFI, wherein said agonist induces the growth of nerves or neurons.

4. The method of claim 3, wherein said agonist induces or stimulates the growth of the axonal projection.

5. The method of claim 3, wherein the agonist enhances or stimulates axonal differentiation.

6. The method of claim 3, wherein the agonist stimulates the growth of the axonal projection between the glossopharyngeal ganglion in the hindbrain.

7. The method of claim 3, wherein the agonist stimulates the growth of the axonal projection between the right and left hemispheres in the thalamus and

8. The method of claim 3, wherein the agonist stimulates growth of the IX ganglion.

9. A method for the prevention of nerve and neuron degeneration in mammals comprising the step of administering to a mammal a therapeutically effective amount of an agonist of COUP-TFI, wherein said agonist enhances nerve or nerve growth.

10. A method for treating learning and memory disorder in a mammal comprising administering to a mammal affected with axonal myelination a therapeutic effective amount of an agonist of COUP-TFI, wherein said agonist induces growth of neurological tissue.

11. A method of treating hearing defects comprising the step of administering to a mammal affected with said hearing defect a therapeutic effect amount of an agonist to COUP-TFI, wherein said agonist induces growth of the inner ear and enhances growth of hair cells allowing restoration of hearing loss.

12. A method of treating balance defects in a mammal comprising the step of administering to a mammal affected with said balance defects therapeutic effective amount of an agonist to COUP-TFI, wherein said agonist induces growth in the inner ear.

13. A method of inducing regeneration of bone formation in a mammal comprising the step of administering to said mammal a therapeutic effective amount of agonist of COUP-TFI, wherein said agonist induces bone formation.

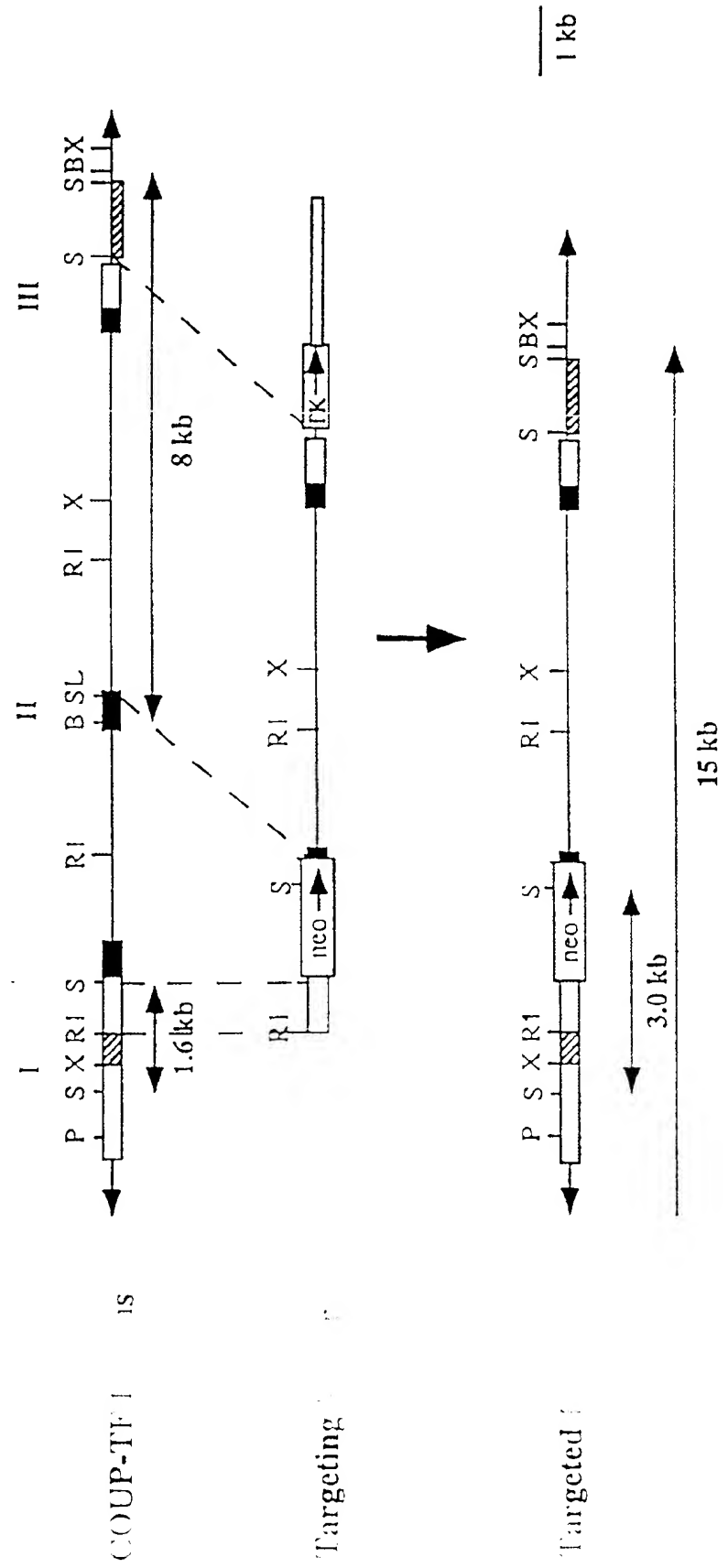
15. A method of treating to the inner ear in a mammal comprising the step of administering to a mammal with an injured inner ear a therapeutic effective amount of an agonist to COUP-TFI, wherein said agonist induces growth or inhibits degeneration of the inner ear.

16. A method for enhancing the growth of the cochlear duct, scala tympani or sacculus in a mammal comprising the step of administering a therapeutic effective amount of an agonist to COUP-TFI, wherein said agonist induces growth.

17. A method for treating neurodegenerative diseases in a mammal comprising the step of introducing by genetic therapy a COUP-TFI gene operatively linked to a promoter, wherein said COUP-TFI gene is inducible by the addition of an agonist and said expression of COUP-TFI enhances expression of COUP-TFI increasing the growth of neural and neuronal tissue.

18. A method for treating bone diseases in a mammal comprising the step of introducing by genetic therapy a COUP-TFI gene operatively linked to a promoter, wherein said COUP-TFI gene is inducible by the addition of an agonist and said expression of COUP-TFI enhances expression of COUP-TFI increasing the growth of bone tissue.

19. A method for treating hearing and balance diseases in a mammal comprising the step of introducing by genetic therapy a COUP-TFI gene operatively linked to a promoter, wherein said COUP-TFI gene is inducible by the addition of an agonist and said expression of COUP-TFI enhances expression of COUP-TFI increasing the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Targeting
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Figure 1

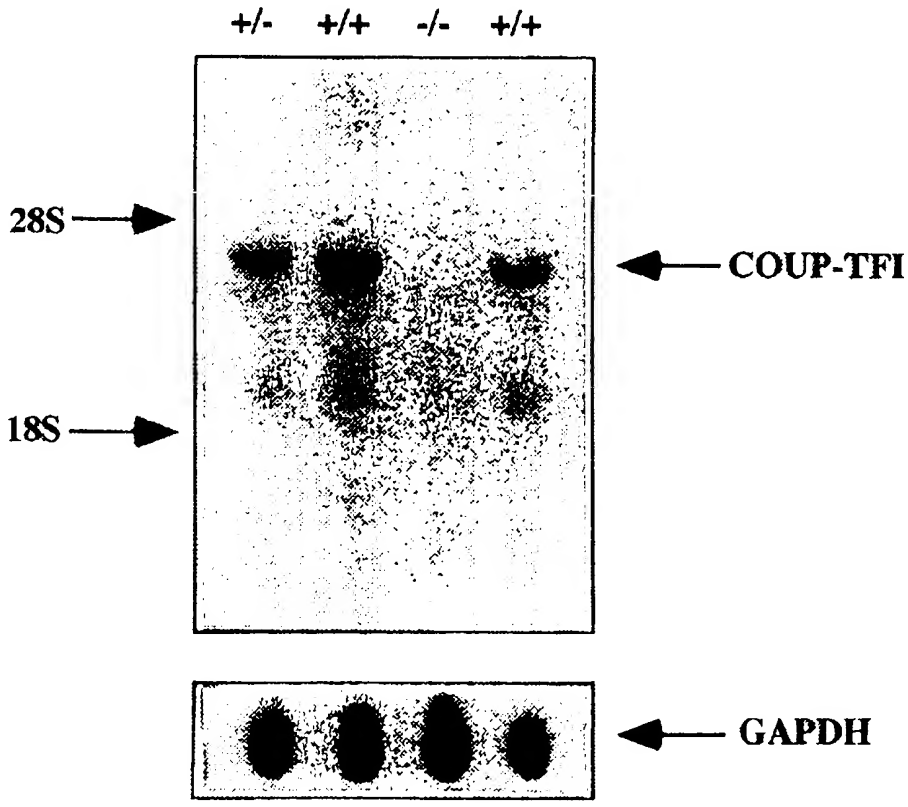


Figure 2A

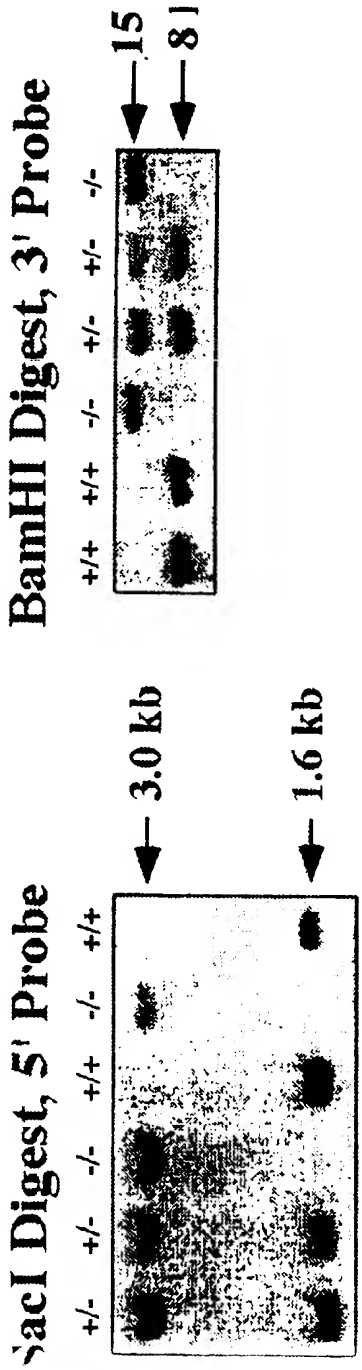


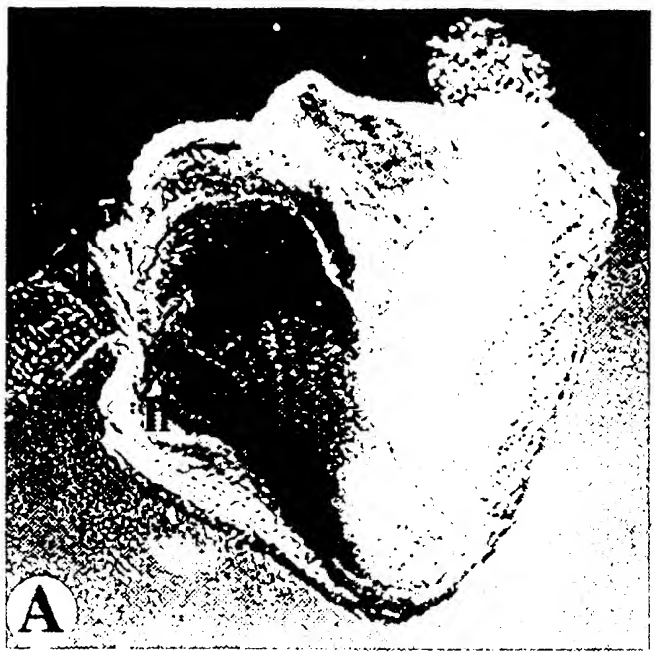
Figure 2B

Figure 3A

Figure 3B

Figure 3C

Figure 3D



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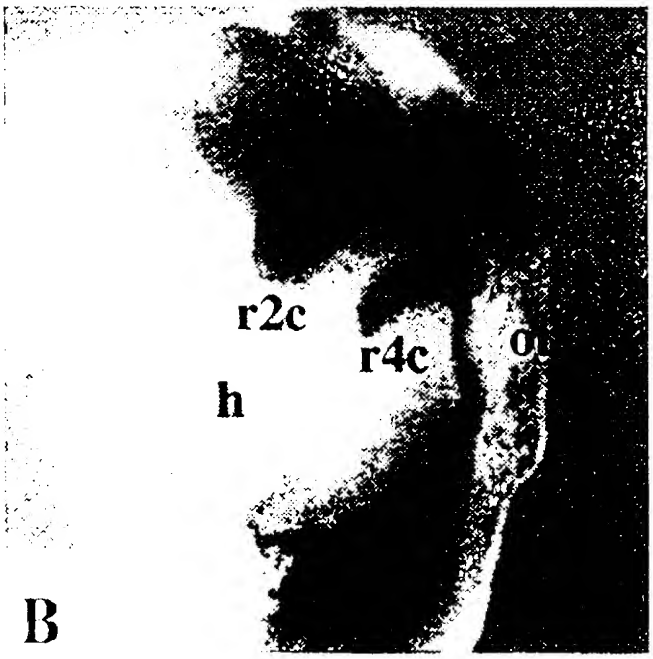


Figure 4B

Figure 4C

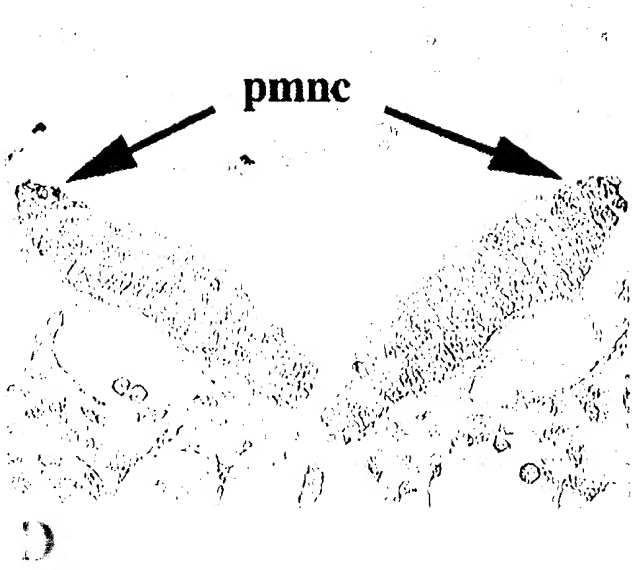


Figure 4D

Figure 5C

Figure 5B

Figure 5A

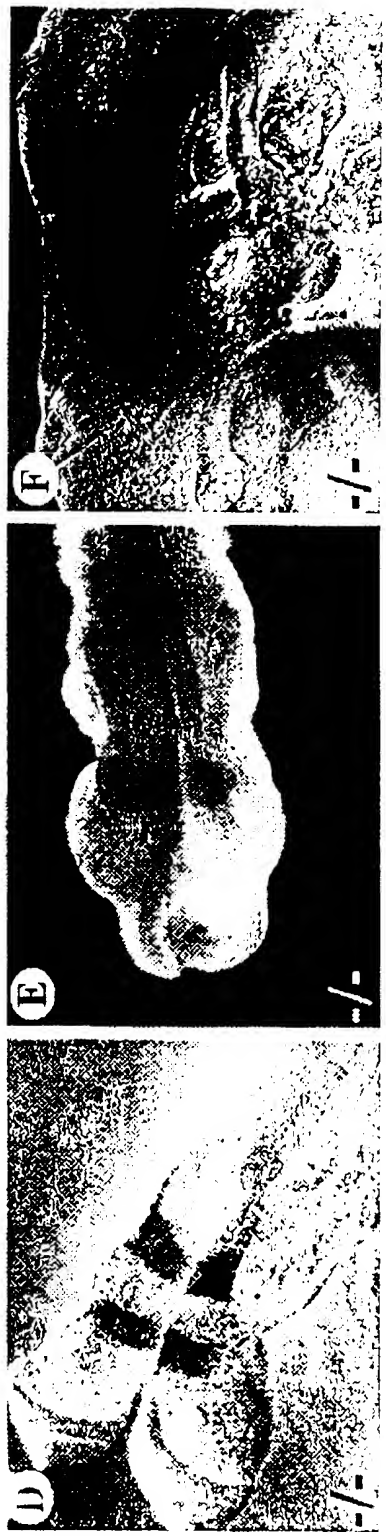


Figure 5D

Figure 5E

Figure 5F

Figure 6C

Figure 6B

Figure 6A

Figure 6F

Figure 6E

Figure 6D

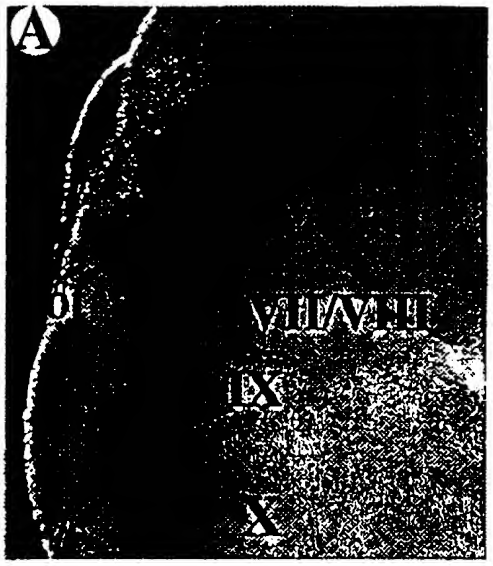


Figure 7A

Figure 7B

Figure 7C

Figure 7D

Figure 8A

Figure 8B

Figure 8C

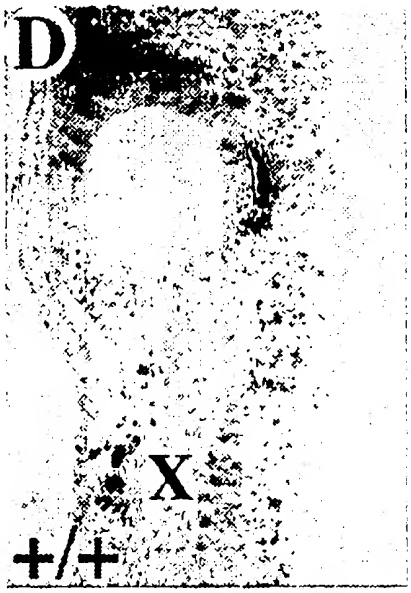


Figure 8D

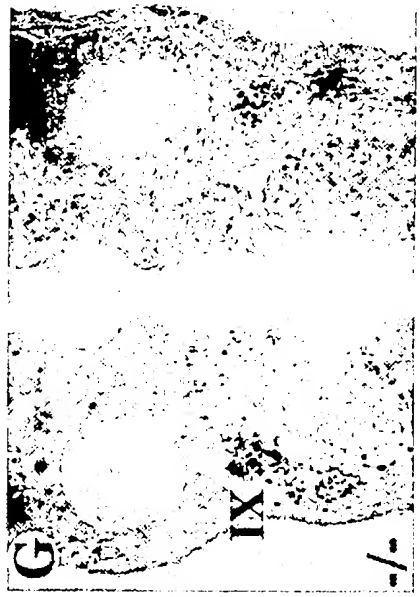


Figure 8G

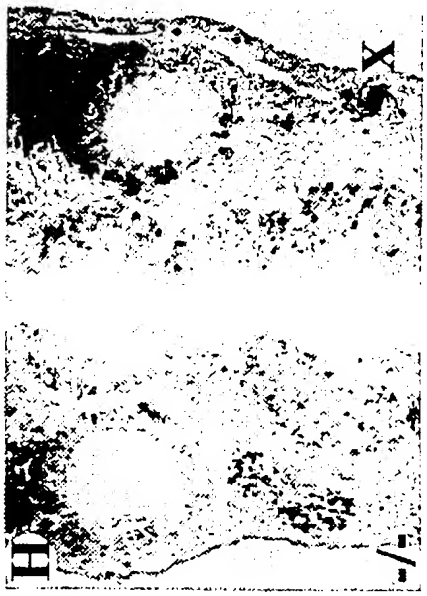
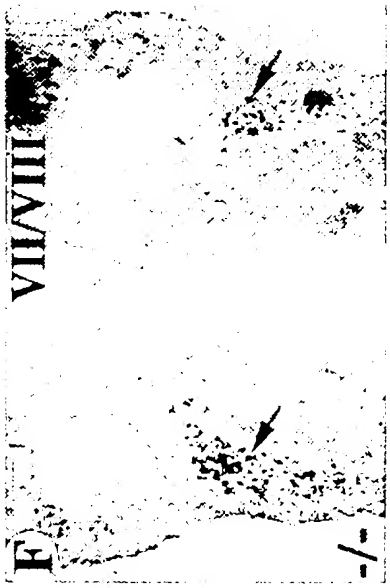


Figure 8H

Figure 8E



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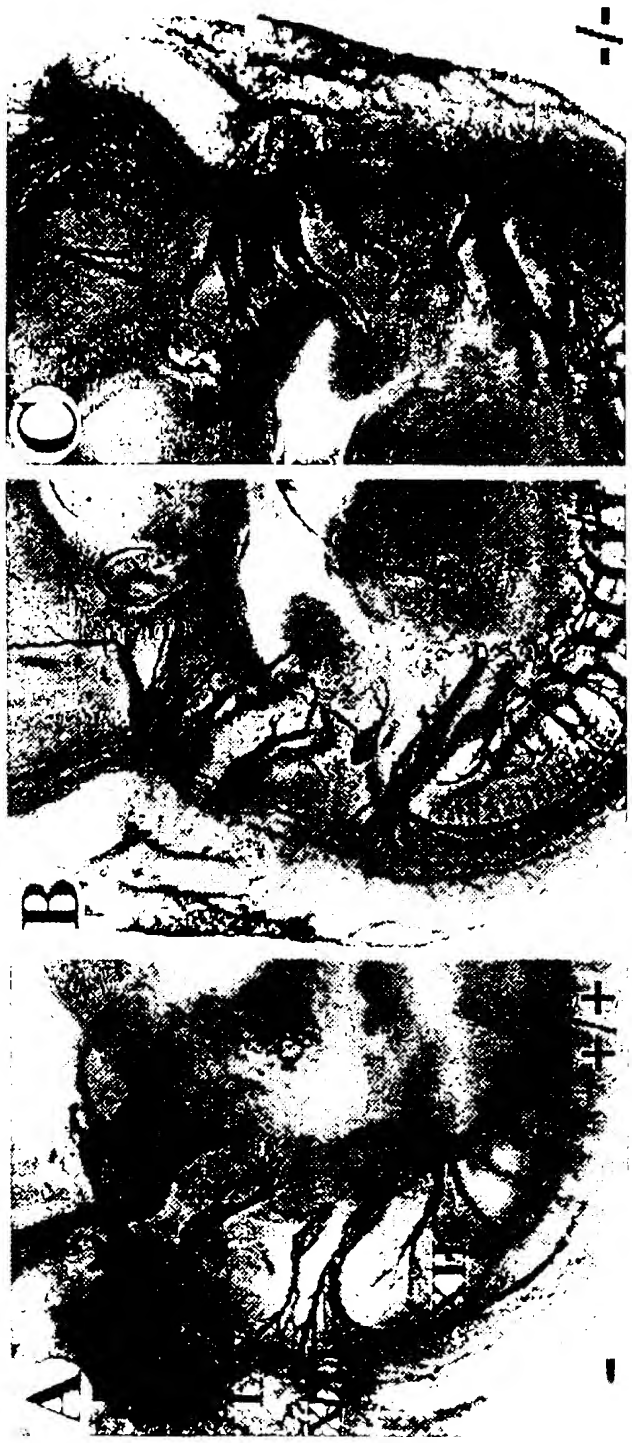


Figure 9C

Figure 9B

Figure 9A

Figure 9F

Figure 9E

Figure 9D

Figure 9I

Figure 9H

Figure 9G

Figure 10B

Figure 10D

Figure 10A

Figure 10C

Figure 11A

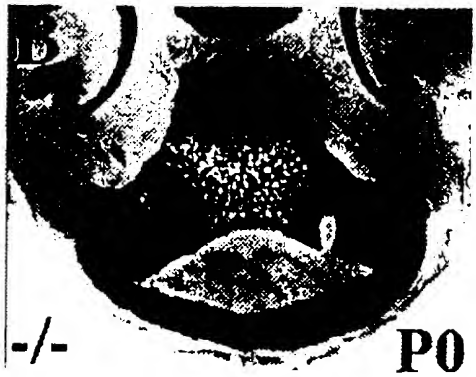


Figure 11B

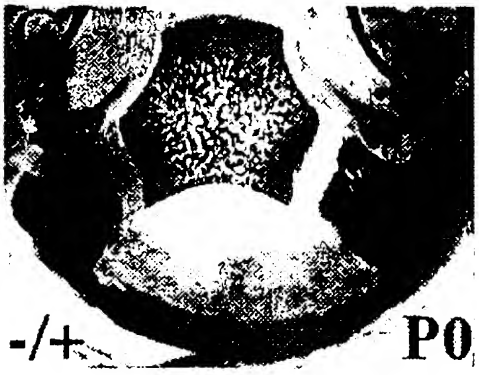


Figure 11C

Figure 11D

Figure 11E

Figure 11F

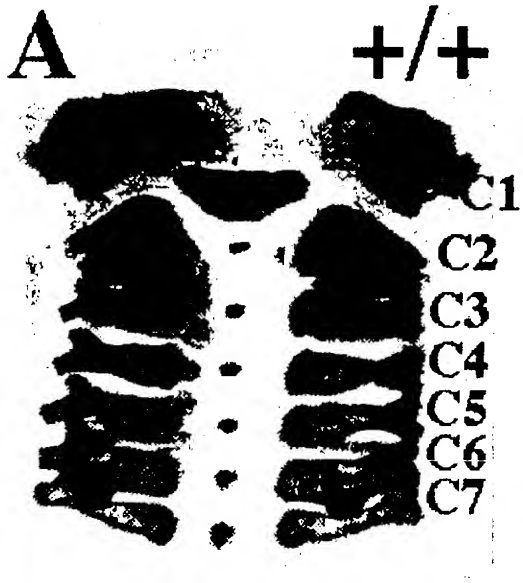
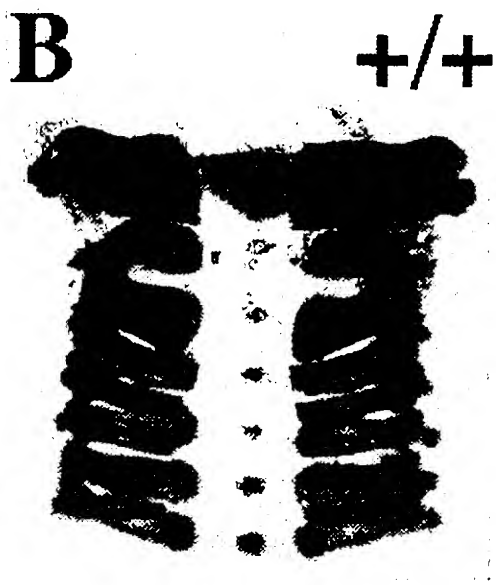


Figure 12A



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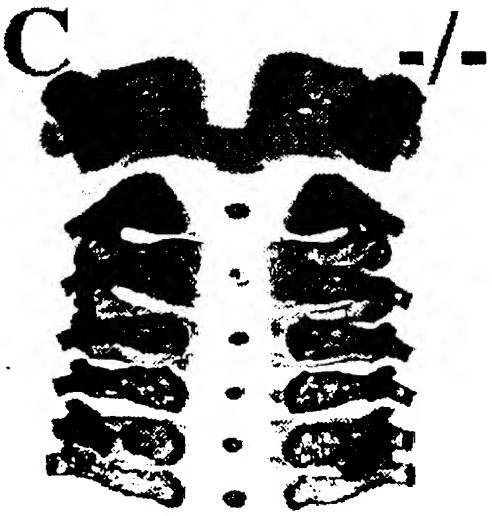


Figure 12C

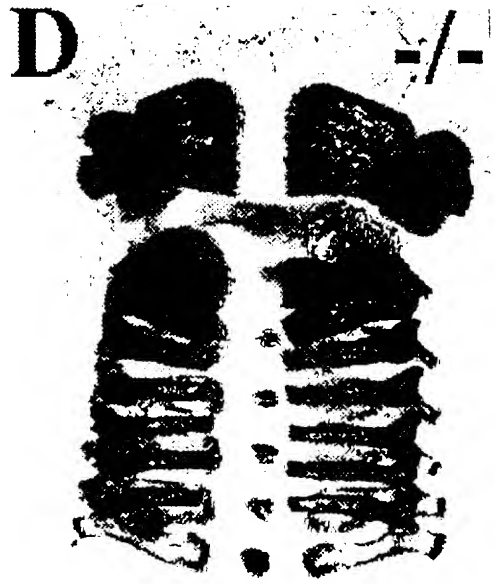


Figure 12D

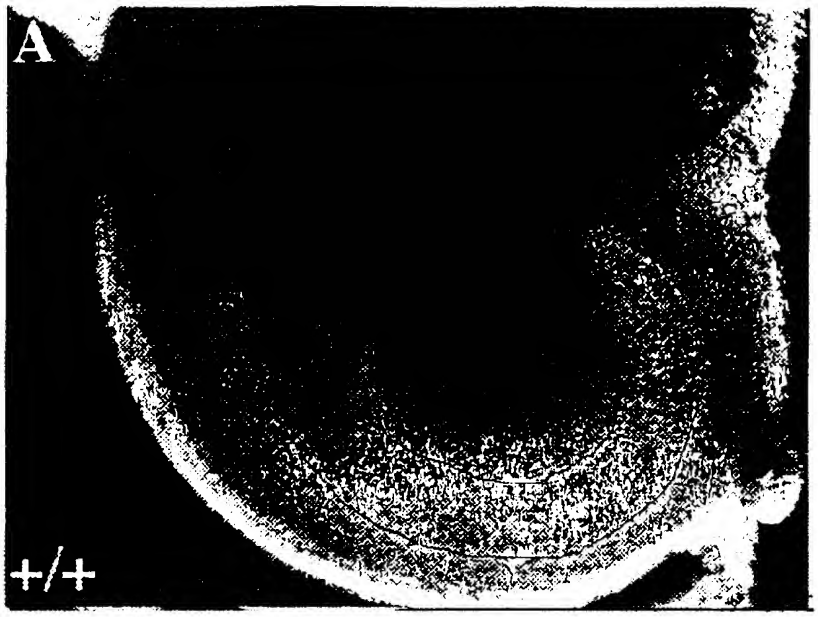


Figure 13A

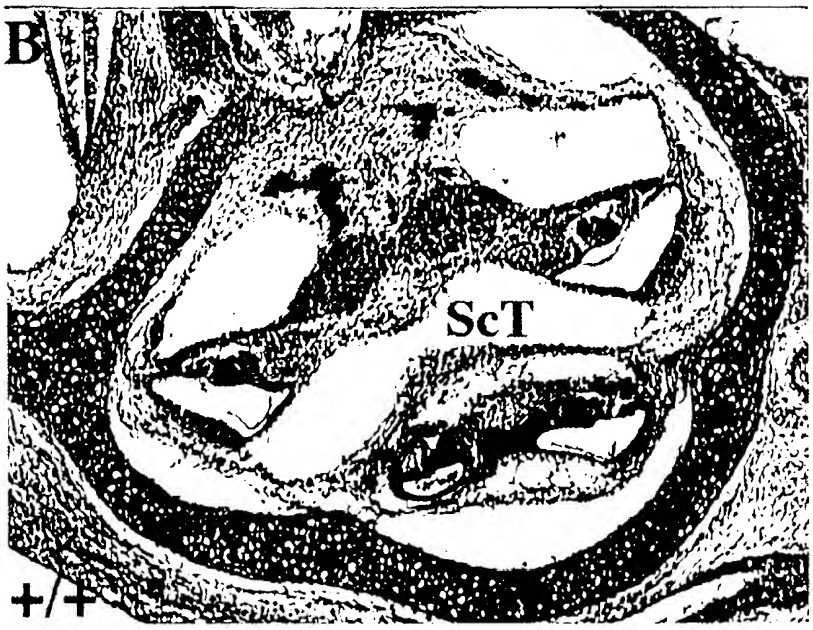


Figure 13B

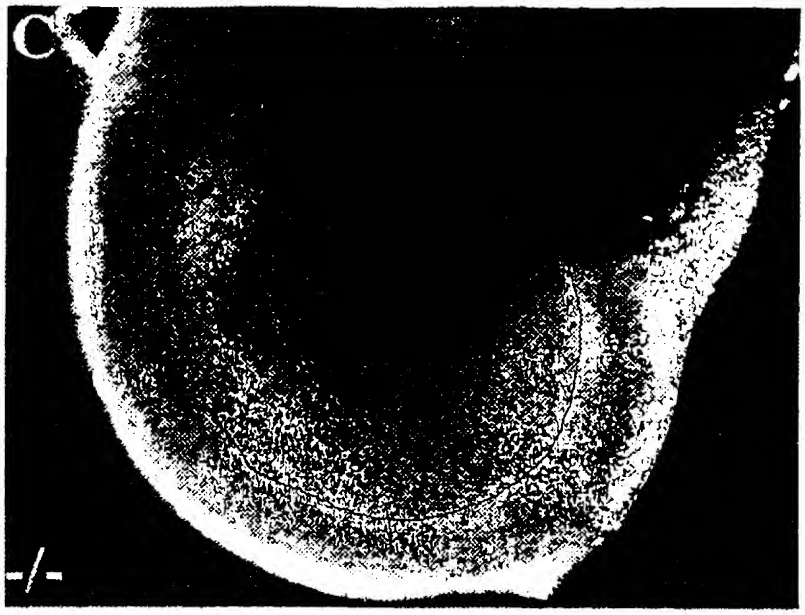
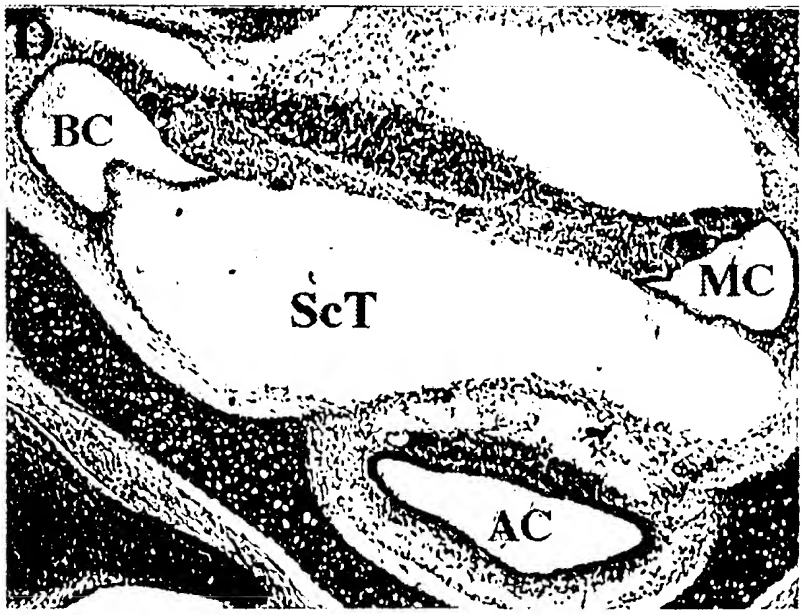


Figure 13C



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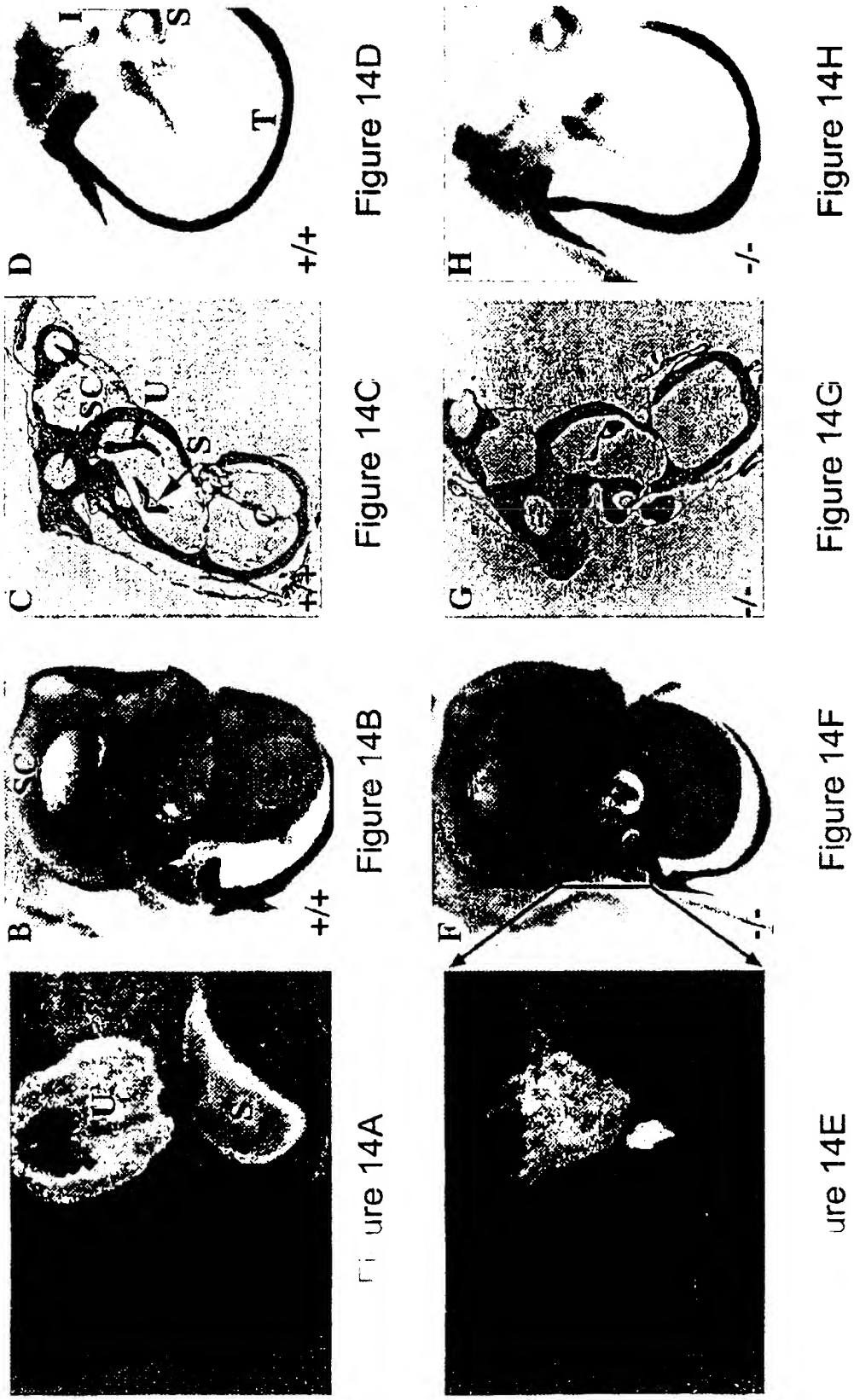


Figure 14A

Figure 14B

Figure 14C

Figure 14D

ure 14E

Figure 14F

Figure 14G

Figure 14H

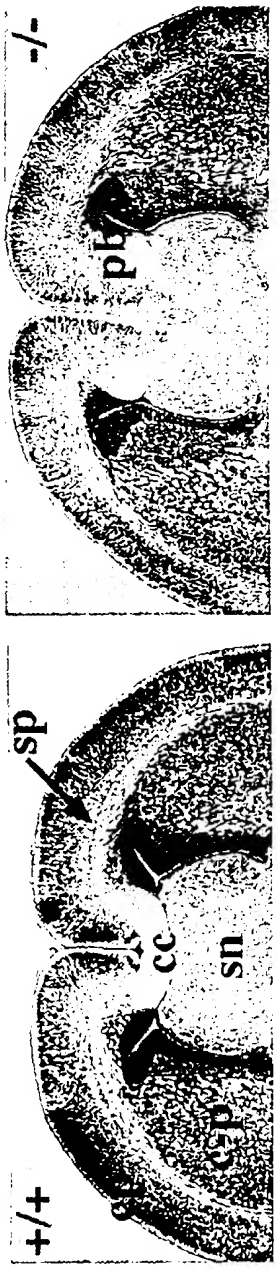


Figure 15A

Figure 15B

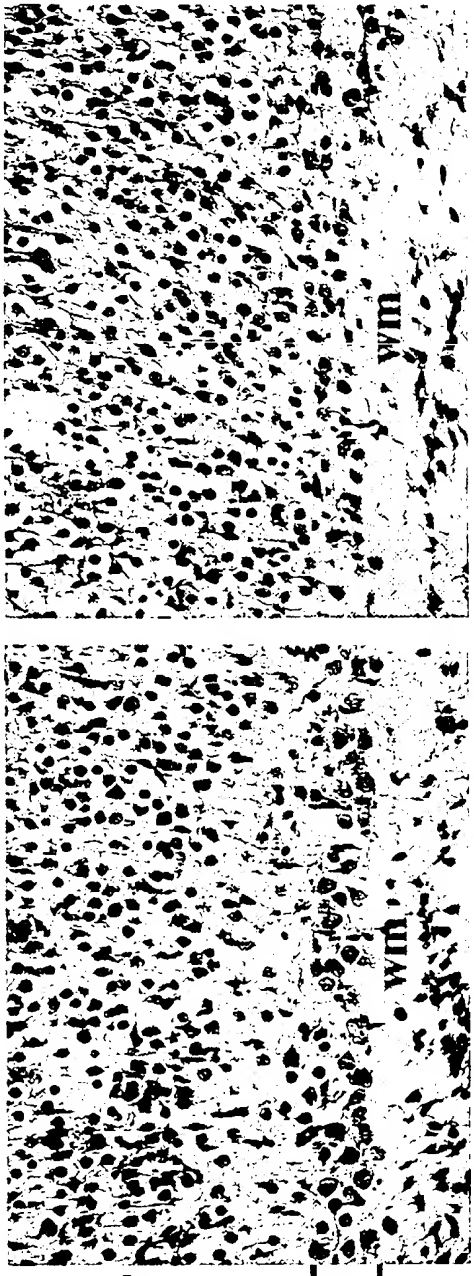
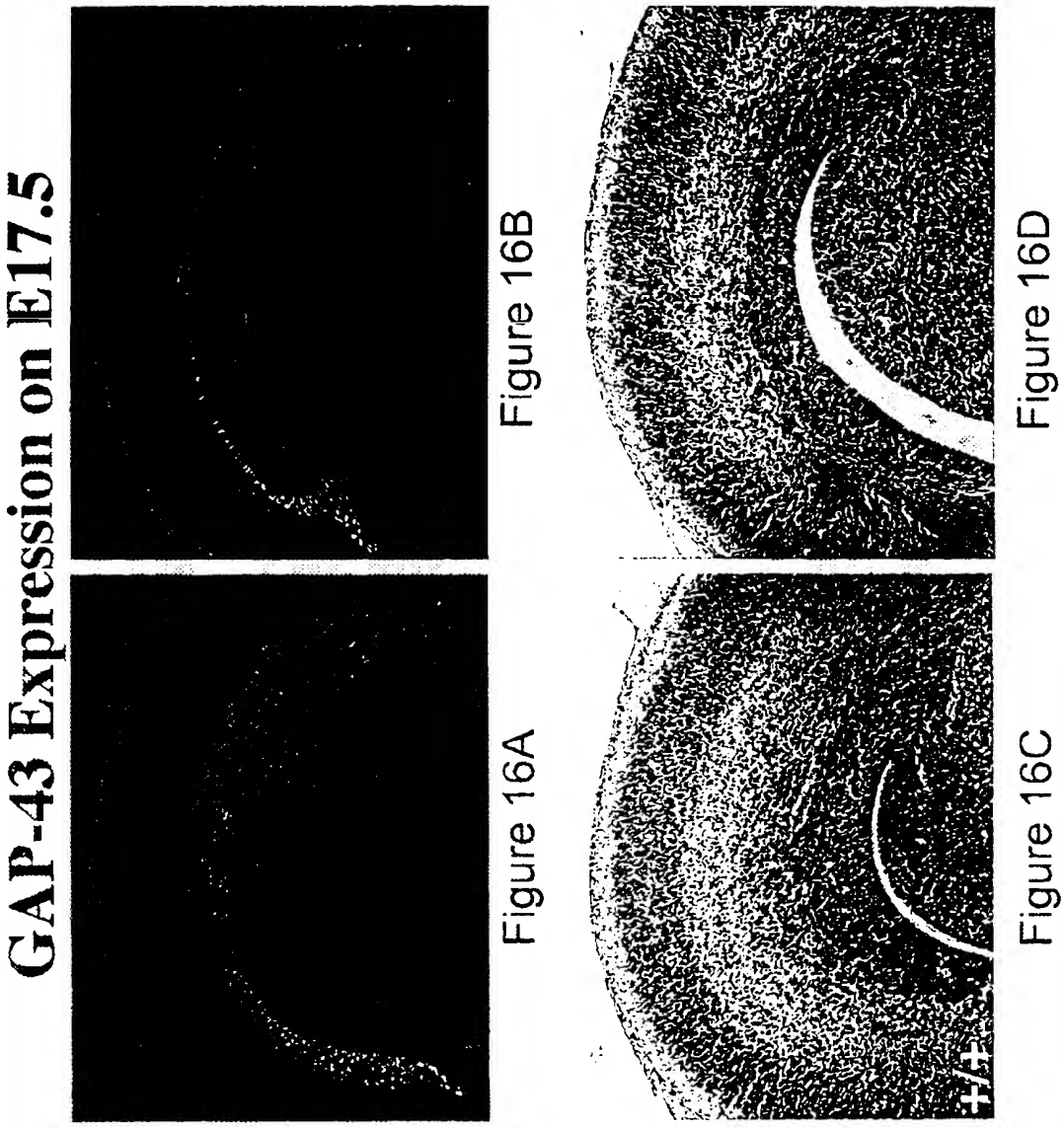


Figure 15C

Figure 15D


GAP-43 Expression on E17.5
Figure 16A
Figure 16B
Figure 16C
Figure 16D

The Cortex of 3-Week COUP-TFI Mice

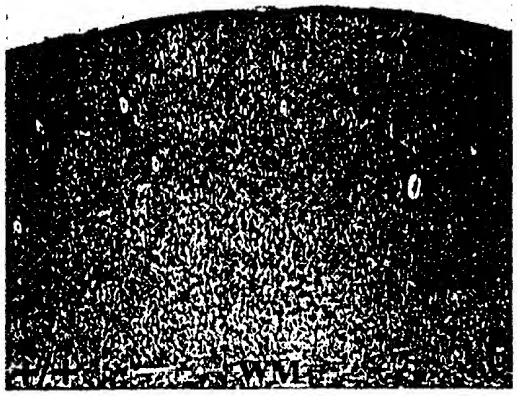


Figure 17A

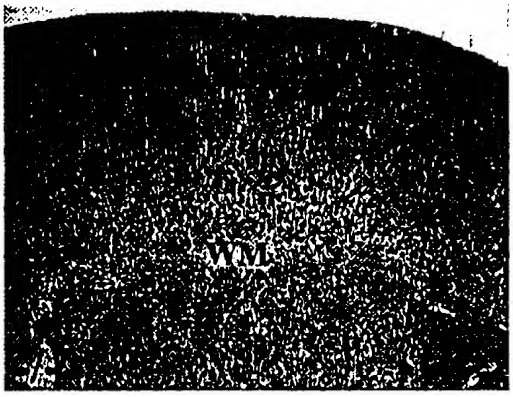


Figure 17B

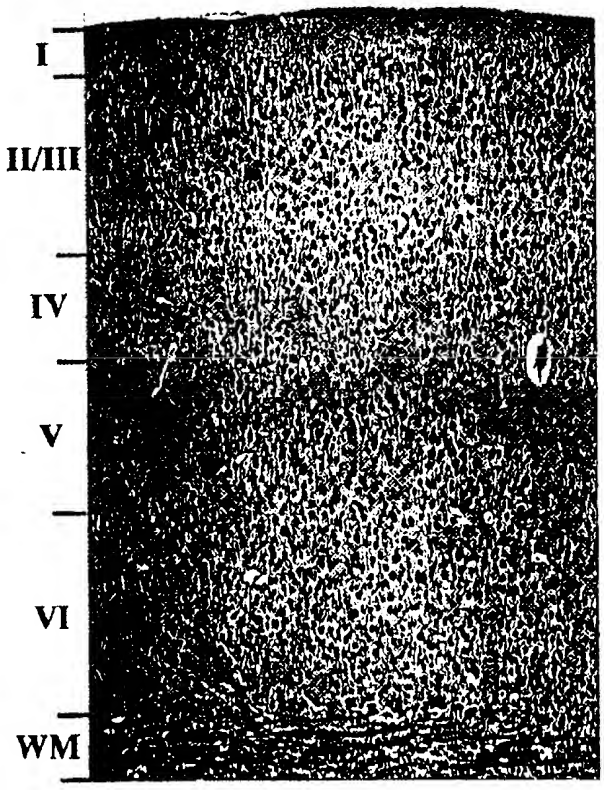


Figure 17C

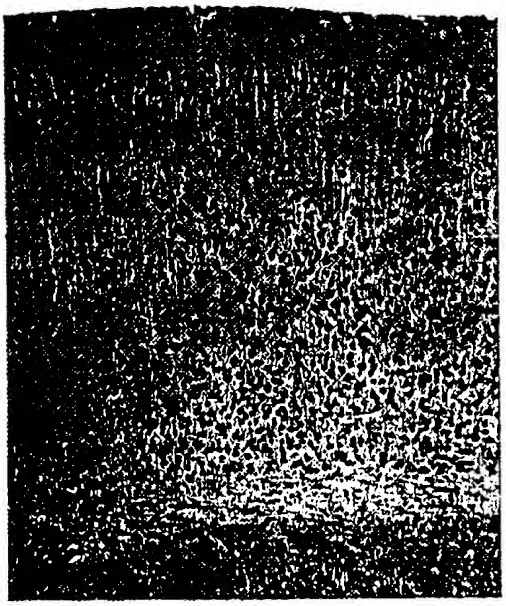


Figure 17D

Lack of Axon Myelination in COUP-TFI Mutant

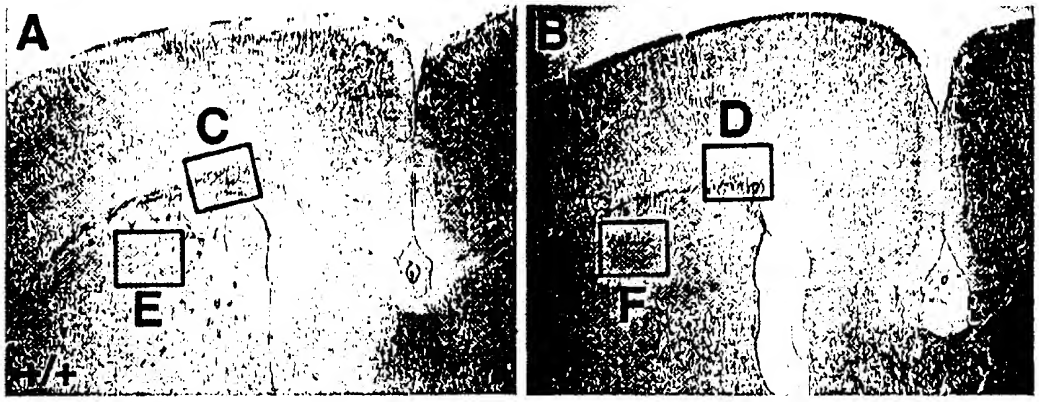


Figure 18A Figure 18B

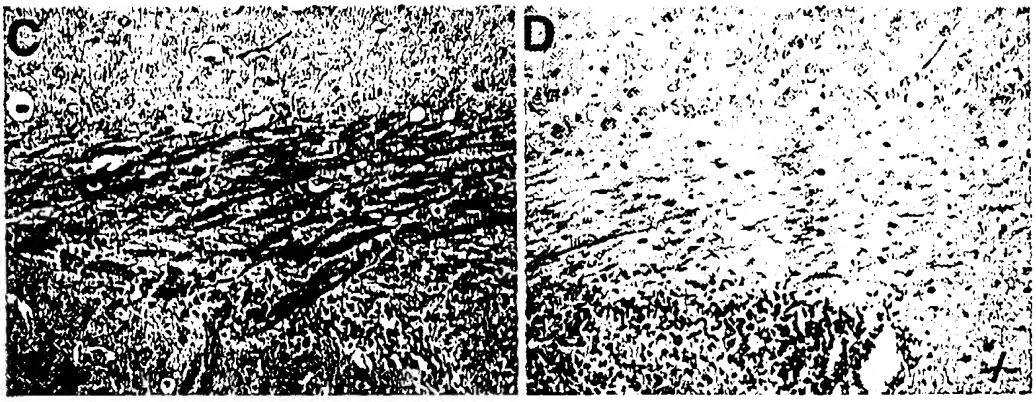


Figure 18C Figure 18D

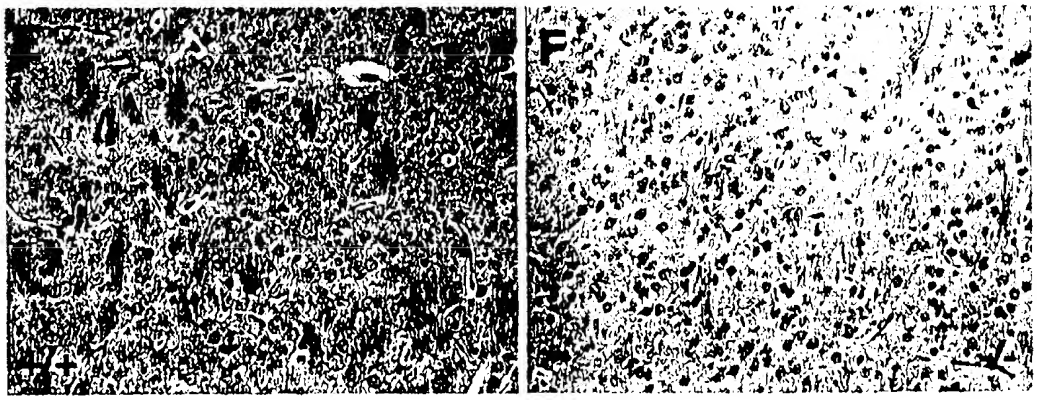


Figure 18E Figure 18F

Decreased Expression of Myelin Basic Protein in COUP-TFI Mutant

Figure 19A

Figure 19B

Figure 19C

Figure 19D

Figure 19E

Figure 19F

Decreased SCIP Expression in COUP-TFI Mutant Cortex

Figure 20A

Figure 20B

Figure 20C

Figure 20D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15737

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/00, 48/00
US CL :514/1, 44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/1, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CHEMICAL ABSTRACTS, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	QUI, Y. et al. Spatiotemporal Expression Patterns of Chicken Ovalbumin Upstream Promoter-Transcription Factors in the Developing Mouse Central Nervous System: Evidence for a Role in Segmental Patterning of the Diencephalon. Proceedings of the National Academy of Sciences (USA). May 1994, Vol. 91, pages 4451-4455, see especially 4453-4455.	1-19
Y	WANG, L-H. et al. Genomic Cloning of COUP Transcription Factor- A Member of the Steroid-thyroid Hormone Receptor Superfamily. Journal of Cellular Biochemistry. 31 March-22 April 1990, Supplement 14, part E, page 112, see abstract.	1-19

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
23 OCTOBER 1998	13 NOV 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DEBORAH CROUCH, PH.D
Facsimile No. (703) 305-3230	Telephone No. (703) 308-[illegible]

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US98/15737

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
Y	QUI, Y. et al. Differential Expression of COUP-TFI and II in Developing Mouse Central Nervous System. Journal of Cellular Biochemistry. 21 January-13 February 1994, Supplement 18B, page 335, see abstract.	1-19
Y	FENG, J.Q. et al. The Mouse Bone Morphogenetic Protein-4 Gene. Journal of Biological Chemistry. 24 November 1995, Vol. 270, No. 47, pages 28364-28373, see especially pages 28370-28372.	1-19